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II. REMARKS

Claims 1 to 3, 5, 13, 14, 20, and 40 to 45 are pending.

Applicants and Applicants' representative gratefully acknowledge the Examiner's careful attention to the application and helpful suggestions made in the telephone interview held April 29, 2004.

The present invention is based, in part, on Applicants' discovery that Activin Type II receptors (Act RII) specifically bind and mediate signal transduction of myostatin (GDF-8), which is a negative regulator of muscle cell growth, and that expression of a dominant negative Act IIR, which lacks kinase activity, in muscle cells inhibits the negative regulatory effect of myostatin, resulting in increased muscle mass in transgenic animals expressing the dominant negative Act IIR. Issues discussed in the interview included 1) whether the disclosure that a transgenic mouse exhibited increased muscle mass due to expression of a truncated Act RII lacking kinase activity would have been predictive of the effect of expressing a truncated Act RII lacking kinase activity in other transgenic mammals; 2) whether the demonstration that expression of a truncated Act RII comprising amino acid residues 1 to 174 of Act RIIB resulted in increased muscle mass would have been predictive of the effect of other truncated Act IIR lacking kinase activity; and 3) whether it would have been predictable that expression of the transgene, following its integration into the genome, will produce the desired phenotype.

As set forth in greater detail below, evidence is provided that the increased muscle mass obtained by expressing a dominant negative Act RIIB in transgenic mice correlates with the increased muscle mass observed in mice and cattle having myostatin (GDF-8) gene mutations, including in GDF-8 knockout mice and in two breeds of double muscling cattle. In view of the knowledge in the art that genetic mutations that inhibit expression of myostatin in diverse

mammals, including cattle and mice, result in increased muscle mass in the various mammals, the skilled artisan, viewing the subject application, would have known that the results disclosed for the transgenic mice expressing a dominant negative Act RII are predictive of results that would be obtained in other transgenic mammals expressing a truncated Act RII lacking kinase activity. Evidence also is provided that one skilled in the art, viewing the subject application, would have known that the increased muscle mass that results due to expression of the dominant negative Act RIIB similarly would have been obtained using a truncated Act RIIA lacking kinase activity. Finally, it is noted that the specification discloses that seven founder animals were identified that tested positive for the truncated Act RIIB transgene, and that all seven exhibited increased skeletal muscle mass (see page 128, paragraph 349; and Table 2, page 133 - "dom. neg. Act RIIB", males and females). Further, while muscle weights varied among the founder animals, the magnitude of the increase was highly consistent in offspring of the founders (see page 128, paragraph 350; and Table 3, page 134).

Rejections under 35 U.S.C. § 112

The objection to the specification and corresponding rejection of claims 1 to 5, 13, 14, 20 and 40 to 45 under 35 U.S.C. § 112, first paragraph, as allegedly lacking enablement is respectfully traversed.

It is acknowledged in the Office Action that the specification is enabling for a transgenic mouse whose genome contains a nucleic acid sequence encoding a truncated Activin IIB receptor ("ActRIIB") consisting of amino acid residues 1 to 174 operably linked to the myosin light chain promoter and 1/3 enhancer, wherein elevated levels of the truncated receptor result in increased muscle mass in the transgenic mouse as compared to a corresponding non-transgenic mouse, and methods of making such a transgenic mouse. It is maintained, however, that the specification is not enabling for all transgenic non-human mammals or for other truncated

Activin Type II receptors expressed from other promoters. Further, it is clarified that the basis of the rejection is that it allegedly would not have been predictable, based on the generation of a single transgenic mouse using a specific construct, that expression of any truncated Act RII lacking kinase activity using any promoter would result in increased muscle mass in any transgenic non-human mammal.

Transgenic Non-Human Mammals

It is submitted that Applicants' disclosure of a transgenic mouse that exhibits increased muscle mass due to expression of a truncated Act RIIB lacking kinase activity is predictive of the effect that would occur in other transgenic non-human mammals expressing a truncated Act RIIB lacking kinase activity because expression of the dominant negative Act RIIB inhibits the effect of myostatin (GDF-8) in the mice, and because it is well known that the loss of myostatin activity in a mammal results in increased muscle mass in the mammal. For example, U.S. Pat. No. 5,994,618 (a copy of which, excluding the Sequence Listing, is attached as Exhibit A) discloses that transgenic GDF-8 knock out mice exhibit increased muscle mass (Example 8, columns 26-28; see, e.g., column 27, lines 27-55). This result indicates that a null mutation of the GDF-8 gene in mice results in increased muscle mass, and demonstrates that inhibition of GDF-8 activity, whether due to a null mutation as disclosed in U.S. Pat. No. 5,994,618 or to expression of a dominant negative Act RIIB receptor as disclosed in the subject application, correlates with increased muscle mass.

Similar to the increased muscle mass observed in the GDF-8 knock out mice (and transgenic mice of the invention), various inactivating mutations of the myostatin (GDF-8) gene in cattle result in the double muscling phenotype, wherein the have increased muscle mass as compared to corresponding normal cattle (see, e.g., McPherron and Lee, *PNAS*, 1997, which is of record in this case; see Figure 2, page 12459). More specifically, a deletion and frameshift

mutation, which "is expected to be a null mutation", results in the loss of most of the mature C-terminal portion of myostatin in Belgian blue cattle, which exhibit the double muscling phenotype (McPherron and Lee, 1997, page 12458, paragraph bridging columns, and paragraph bridging pages 12458-12459). In addition, a point mutation that results in the substitution of tyrosine for an invariant cysteine residue, and "is likely to result in complete or almost complete loss of function", occurs in Piedmontese cattle, which also are characterized by the double muscling phenotype (McPherron and Lee, 1997, page 12459, paragraph bridging columns). These results indicate that myostatin generally acts as a negative regulator of muscle growth in mammals, and that a loss of myostatin activity correlates with increased muscle mass.

In summary, the results of Exhibits A and B demonstrate that GDF-8 (myostatin) generally functions as a negative regulator of muscle growth in mammals, and that genetic mutations that result in loss of GDF-8 activity are correlated with increased muscle mass in different mammalian species (see, also, Lee and McPherron, 1997, last sentence of Abstract, suggesting the results can be extended to "other farm animals"). Further, as disclosed in the specification, transgenic mice expressing an Act RIIB lacking kinase activity, like mice having a GDF-8 gene null mutation, exhibited increased muscle mass, thus demonstrating that increased muscle mass occurs in mice, regardless of whether GDF-8 activity is inhibited due to a genetic mutation or to expression of a dominant negative GDF-8 receptor (Act RIIB). Accordingly, it is submitted that the skilled artisan would have known that the increased muscle mass obtained in the exemplified transgenic mice is predictive of the effect that would occur in other transgenic mammals expressing such a truncated Act RIIB because the increased muscle mass occurs in mice expressing a dominant negative Act RIIB correlates with the increased muscle mass observed in mice having a GDF-8 gene mutation (Exhibit A), which, in turn, correlates with the increased muscle mass observed in cattle having a GDF-8 (myostatin) gene mutation (Exhibit B).

Activin Type II Receptors

It is also submitted that the skilled artisan would have known that the result obtained by expressing a truncated Act RIIB lacking kinase is predictive of the result that would be obtained by expressing other such Activin Type II receptors such as a truncated Act RIIA lacking kinase activity because GDF-8 (myostatin) specifically binds Act RIIA and Act RIIB, and because deletion of the kinase domain of the Activin Type II receptors, which are structurally related, results in dominant negative activity of the truncated receptors. In this respect, Lee and McPherron (*PNAS* 98:9306, 2001, a copy of which is attached as Exhibit B) report that myostatin (GDF-8) binds to both Act RIIA and Act RIIB, and suggest that myostatin signal transduction is mediated by Activin Type II receptor binding (see Abstract; and page 9307, Figure 1C; page 9311, left column, first full paragraph).

Matthews and Vale (U.S. Pat. No. 5,885,794, which is of record in this case) describe Activin Type II receptors, which are a subclass of Activin receptors that share greater than 80% amino acid sequence homology (see column 4, lines 10-45). A schematic diagram of Act RII is provided in Figure 1 of U.S. Pat. No. 5,885,794, and illustrates the C-terminal kinase domain. The initially identified Activin Type II receptor, designated "Act RII", was used to identify a human homolog ("Human Activin Receptor A"; see column 15, lines 33-48). The Activin Type II receptors referred to as "Act RII" in U.S. Pat. No. 5,885,794 also are known as Activin Type IIA receptors (Act RIIA), and are referred to as Act RIIA in the subject application (see, also, "Swiss-Prot" report, attached as Exhibit C).

The Tsuchida et al. reference (*Endocrinology* 136:5493, 1995, a copy of which is attached as Exhibit D), which includes W.W. Vale (see U.S. Pat. No. 5,885,794) as an author, describes a truncated "Act RII", which lacks kinase activity and includes a hemagluttinin (HA) tag ("ΔActRII(HA)"; see page 5494, paragraph bridging columns; and paragraph bridging

pages 5498-5499). It is noted that the Act RII used by Tsuchida et al. to generate ΔActRII(HA) corresponds to Act RIIA. As such, like the truncated Act RIIB used to generate the transgenic mice exemplified in the subject application, Tsuchida et al. demonstrate that deletion of the C-terminal kinase domain-containing portion of Act RIIA generates a receptor having dominant negative activity. Accordingly, it is submitted that the skilled artisan, viewing Applicants' disclosure that a transgenic mouse expressing a truncated Act RIIB lacking kinase activity exhibits increased muscle mass, would have predicted that expression of any truncated Activin Type II receptor lacking kinase activity would result in increased muscle mass in a transgenic mammal because it was known that Activin Type II receptors share a highly conserved sequence homology, including a conserved kinase domain (see U.S. Pat. No. 5,885,794), and because a truncated Act RIIA that lacks kinase activity and has dominant negative activity was known (Exhibit D).

Transgene Expression and Phenotype

It is further submitted that, even if expression of a transgene encoding a truncated Act RII lacking kinase activity may vary depending, for example, on the position in the genome in which it inserts, the skilled artisan, viewing the subject application, would have known that any level of expression of the dominant negative Act RII would likely to result in some increase in muscle mass in the transgenic mammals. The claims require that the transgene is "expressed so as to result in elevated levels of the truncated Activin Type II receptor and increased muscle mass in the transgenic mammal...as compared to a corresponding nontransgenic mammal". As such, the claims do not require any particular level of truncated Activin Type II receptor expression or increased muscle mass, only that the increased muscle mass be measurable when compared with a corresponding nontransgenic mammal.

The specification discloses that, following pronuclear injection of the exemplified transgene, seven founder animals were identified that tested positive for the truncated Act RIIB transgene, and all seven exhibited an increase in skeletal muscle mass as compared to controls (see page 128, paragraph 349; and Table 2, page 133 - panels labeled "dom. neg. Act RIIB", males and females). As is evident from Table 2, the amount of increased muscle mass varies in the different founder animals, though each shows increased muscle mass. As such, assuming the transgene integrated in different positions in the genomes of the different founder animals, the results in Table 2 indicate that, regardless of where in the genome a transgene may insert, the encoded truncated Act RII lacking kinase activity can be expressed, resulting in some level of increased muscle mass. Further, offspring of each founder animals exhibited consistent increases in muscle mass; for example, the muscles of male and female offspring from the C5 founder weighed 30-60% more than controls, and the muscles of male and female offspring of the C11 founder weighed 110-180% more than controls (page 128, paragraph 350; and Table 3, page 134). These results demonstrate that, even if different levels of the dominant negative Act RII are expressed due, for example, to a position effect in the genome, the transgenic mammals nevertheless will exhibit a measurable increase in muscle mass, and will be able to transfer the phenotype to their offspring.

For the reasons set forth above, it is submitted that the specification fully enables the claimed subject matter. In the following discussion, some remaining issues in the Office Actin are addressed.

As discussed in the Amendment filed in response to the previous Office Action, the specification discloses methods of producing transgenic non-human mammals, referring, for example, to U.S. Pat. Nos. 6,140,552 and 6,218,596 (see paragraph 138, page 52-53; and paragraphs 140-143). In addition, U.S. Pat. Nos. 6,271,436 (describing cells and methods for

generating transgenic pigs), 6,107,543 (describing cells and methods for making transgenic bovines), and 6,194,635 (describing cells and methods for making chimeric and transgenic ungulates, particularly porcine species), each of which have a priority before that of the subject application, were submitted as evidence that the skilled artisan, viewing the subject application, would have known how to make a transgenic non-human mammal as claimed, without undue experimentation.

Mullins et al. is cited in the Office Action as stating that "a given construct may react very differently from one species to another" (Summary). As discussed in the Amendment filed in response to the previous Office Action, however, Mullins et al. go on to state, for example, that the application of transgenics in pigs should produce major advances in the fields of transfusion and transplantation (Summary), and indicate that various potential problems that may occur as a result of transgenic methods can be addressed, including, for example, pointing out that "Position-independent, copy-number related expression can be achieved" using a variety of promoter elements and that "Such elements have been shown to function across species barriers, and their incorporation into gene constructs can overcome position effects and improve expression of heterologous genes within specific cell types" (paragraph bridging pages 1557-1558). In addition, Mullins et al. describe several successful applications of transgenics in mammals other than mice (see pages 1558-1559, section entitled "Nonmurine species in biomedical research"). As such, Mullins et al. do not necessarily support the position that undue experimentation would have been required for one skilled in the art to practice the claimed invention at the time the subject application was filed.

McPherron et al. is cited as reporting that "Unlike in mice, a myostatin null mutation in cattle causes a reduction in sizes in internal organs and only a modest increase in muscle mass." As discussed above, however, the claims do not require any particular level of increased muscle

mass. Also, as discussed above, it is submitted that McPherron et al. support enablement of the claimed invention with respect to mammals other than mice because mutations in the myostatin gene of cattle, like a null mutation of the GDF-8 gene in mice, result in increased muscle mass, thus providing objective evidence that the increased muscle mass obtained in the transgenic mice expressing the dominant negative Act RIIB is predictive of the effect that will occur in other transgenic mammals expressing such a dominant negative Act RII.

Yamaoka et al. is cited as teaching that the Activin receptor may be dispensable for normal development of pancreatic islet cells, but that while redundancy may make a particular member of the TGF-β superfamily dispensable in some cases, such a result does not extend to all types of alterations in the TGF-β superfamily, or phenotypes produced by such alterations. It is stated, for example, that Yamaoka et al. report that two alterations of TGF-β (i.e., over-expression of a TGF-β dominant negative, and a TGF-β knockout), which allegedly should result in the same phenotype, result in different effects on pancreatic acinar cells (citing to middle of page 300). As discussed in the interview with the Examiner, however, a review of page 300 (and the entire Yamaoka et al. reference) did not find any mention of experiments relating to a TGF-β knockout phenotype. Instead, the cited passage appears to refer only to results obtained in an experiment using a TGF-β receptor dominant negative construct, and does not appear to provide any indication as to an unexpected or unpredictable phenotype observed due to expression of the dominant negative receptor. Nevertheless, as discussed above, the result obtained due to expression of a dominant negative Act RII in mice correlates with the results obtained in GDF-8 knock out mice. As such, even if Yamaoka et al. report an inconsistency with respect to TGF-β, it would not appear to be relevant to the present invention.

Wall (1996) is cited as reporting that transgene expression in the mouse is not always predictive of the physiological consequences of transgene expression in livestock. However, as

discussed in the previous Amendment, Wall et al. (J. Dairy Sci. 80:2213, 1997) later stated that the "Characteristics of transgene expression in transgenic goats, mice, pigs, rabbits, rats, and sheep appear to be similar. Although data are insufficient to characterize transgene expression for transgenic cattle, there is no reason to expect that transgenes behave differently in that species." (page 2216, left column, first full paragraph). As such, it is submitted that, after the publication of Wall (1996), and prior to the time the subject application was filed, Wall et al. (1997) considered transgene expression in mice to be reasonably predictable of transgene expression in other mammals.

For the above reasons, and those discussed in the interview with the Examiner, and set forth in Applicants' Amendment filed in response to the previous Office Action, it is submitted that one skilled in the art, viewing the subject application, would have known how to make and use the claimed transgenic non-human mammals and practice the methods of the invention without undue experimentation. Accordingly, it is respectfully requested that the Examiner reconsider and withdraw the objection to the specification, and remove the corresponding rejection of the claims under 35 U.S.C. § 112, first paragraph.

In view of the foregoing remarks and Exhibits A to D, it is submitted that the claims are in condition for allowance, and a notice to that effect is respectfully requested. The Examiner is invited to contact Applicants' undersigned representative if there are any questions relating to the subject application.

Enclosed is Check No. 559176 in the amount of $55.00 in payment of the One (1) Month extension of time fee. The Commissioner is hereby authorized to charge any other fees that may be associated with this communication, or credit any overpayment, to Deposit Account No. 50-1355.

Respectfully submitted,

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Enclosures: Exhibits A, B, C and D

Regulation of myostatin activity and muscle growth

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Myostatin is a transforming growth factor-β family member that acts as a negative regulator of skeletal muscle mass. To identify possible myostatin inhibitors that may have applications for promoting muscle growth, we investigated the regulation of myostatin signaling. Myostatin protein purified from mammalian cells consisted of a noncovalently held complex of the N-terminal propeptide and a disulfide-linked dimer of C-terminal fragments. The purified C-terminal myostatin dimer was capable of binding the activin type II receptors, Act RIIB and, to a lesser extent, Act RIIA. Binding of myostatin to Act RIIB could be inhibited by the activin-binding protein follistatin and, at higher concentrations, by the myostatin propeptide. To determine the functional significance of these interactions *in vivo*, we generated transgenic mice expressing high levels of the propeptide, follistatin, or a dominant-negative form of Act RIIB by using a skeletal muscle-specific promoter. Independent transgenic mouse lines for each construct exhibited dramatic increases in muscle mass comparable to those seen in myostatin knockout mice. Our findings suggest that the propeptide, follistatin, or other molecules that block signaling through this pathway may be useful agents for enhancing muscle growth for both human therapeutic and agricultural applications.

Myostatin is a transforming growth factor-β (TGF-β) family member that plays an essential role in regulating skeletal muscle growth (1). Myostatin is expressed initially in the myotome compartment of developing somites and continues to be expressed in the myogenic lineage throughout development and in adult animals. Mice carrying a targeted deletion of the myostatin gene have a dramatic and widespread increase in skeletal muscle mass. Individual muscles of myostatin null mice weigh approximately twice as much as those of wild-type mice as a result of a combination of muscle fiber hyperplasia and hypertrophy. The myostatin sequence has been highly conserved through evolution (2). Remarkably, the human, rat, murine, porcine, turkey, and chicken myostatin sequences are identical in the biologically active C-terminal portion of the molecule following the proteolytic processing site. The function of myostatin also appears to be conserved across species, as mutations in the myostatin gene have been shown to result in the double muscling phenotype in cattle (2–5).

These findings have raised the possibility that pharmacological agents capable of blocking myostatin activity may have applications for promoting muscle growth in human disease settings as well as in livestock animals. To identify novel strategies for blocking myostatin activity, we investigated the regulation of myostatin signaling. Here, we present evidence that myostatin, like TGF-β, may normally exist *in vivo* in a latent complex with the propeptide (the portion of the precursor protein upstream of the proteolytic processing site) and that on activation, myostatin may signal by binding to activin type II receptors.

Materials and Methods

Purification of Myostatin. A Chinese hamster ovary cell line carrying amplified copies of a myostatin expression construct (1) was transfected with an expression construct for the furin protease PACE (kindly provided by Monique Davies, Genetics Institute, Cambridge, MA) to improve processing of the precursor protein. Conditioned medium (prepared by Cell Trends, Middletown, MD) was passed successively over hydroxylapatite (eluted with 200 mM sodium phosphate, pH 7.2), lentil lectin Sepharose (eluted with 50 mM Tris, pH 7.4/500 mM NaCl/500 mM methyl mannose), DEAE agarose (collected flowthrough), and heparin Sepharose (eluted with 50 mM Tris, pH 7.4/200 mM NaCl). The heparin eluate was then bound to a reverse-phase C4 HPLC column and eluted with an acetonitrile gradient in 0.1% trifluoroacetic acid. Antibodies directed against the mature C-terminal protein have been described (1). To raise antibodies against the propeptide, the portion of the human myostatin protein spanning amino acids 122–261 was expressed in bacteria by using the RSET vector (Invitrogen) and purified by nickel chelate chromatography. Immunization of rabbits was carried out by Spring Valley Labs (Woodbine, MD).

Receptor Binding. Purified myostatin was radioiodinated by using the chloramine T method (6). COS-7 cells grown in 6- or 12-well plates were transfected with 1–2 μg pCMV5 or pCMV5/receptor construct by using lipofectamine (GIBCO). Crosslinking experiments were carried out as described (7). For quantitative receptor-binding assays, cell monolayers were incubated with labeled myostatin (in PBS containing 1 mg/ml of BSA) in the presence or absence of unlabeled myostatin, propeptide, or follistatin at 4°C. Cells were then washed, lysed in 0.5 M NaOH, and counted in a gamma counter. Specific binding was calculated as the difference in bound myostatin between cells transfected with Act RIIB and cells transfected with vector. This method of calculating specific binding was especially important in assessing the effect of the propeptide as the addition of the propeptide also reduced nonspecific binding in a concentration-dependent manner. Recombinant human follistatin was obtained through the National Hormone and Pituitary Program, the National Institute of Diabetes and Digestive and Kidney Diseases, and A. F. Parlow (Harbor–University of California, Los Angeles, Medical Center, Torrance, CA).

Transgenic Mice. DNAs encoding a truncated form of murine Act RIIB (amino acids 1–174), the murine myostatin propeptide (amino acids 1–267), and the human follistatin short form were cloned into the MDAF2 vector containing the myosin light chain promoter and 1/3 enhancer and simian virus 40 processing sites (8). All microinjections and embryo transfers were carried out by the Johns Hopkins School of Medicine Transgenic Core Facility. Transgenic founders in a hybrid SJL/C57BL/6 background were mated to wild-type C57BL/6 mice, and all studies were carried out by using F1 offspring. For analysis of muscle weights, individual muscles were dissected from both sides of nearly all animals, and the average of the left and right muscle weights was used. Analysis of fiber numbers and sizes was carried out as described (1). RNA isolation and Northern analysis were carried out as described (9).

Results

To overproduce myostatin protein, we generated a Chinese hamster ovary cell line carrying amplified copies of a myostatin expression construct. We purified myostatin protein from the conditioned medium of this cell line by successive fractionation

Abbreviations: TGF-β, transforming growth factor-β; BMP, bone morphogenetic protein; GDF-11, growth/differentiation factor-11.

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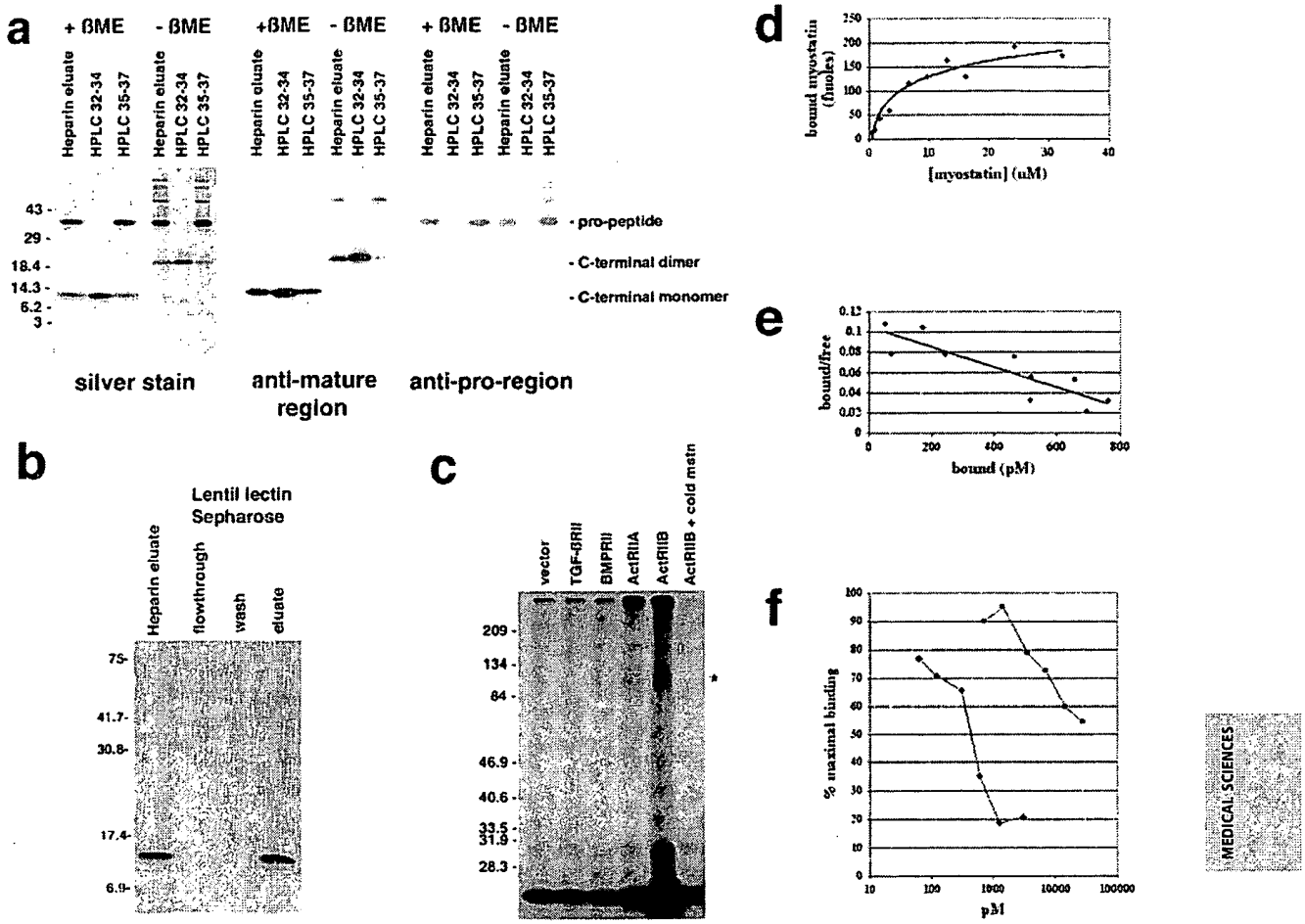

Fig. 1. Binding of myostatin to activin type II receptors. (*a*) Analysis of purified myostatin protein. Myostatin protein preparation following the heparin column (heparin eluate) or following reverse-phase HPLC (fractions 32–34 or 35–37 containing the C-terminal region or propeptide, respectively) was electrophoresed under reducing (+βME) or nonreducing (−βME) conditions and either silver stained or subjected to Western analysis. (*b*) Binding of myostatin to lentil lectin. The heparin eluate was bound to lentil lectin Sepharose and eluted with methyl mannose. Samples were electrophoresed under reducing conditions, blotted, and probed with antibodies directed against the C-terminal region. Similar analysis by using antibodies directed against the pro region showed that the pro region was also retained on the column and eluted with methyl mannose (data not shown). (*c*) Crosslinking experiments. COS-7 cells transfected with expression constructs for the indicated receptors were incubated with ^{125}I-myostatin followed by the crosslinking agent disuccinimidyl suberate. Crosslinked complexes were analyzed by SDS/PAGE. Asterisk denotes predicted size for myostatin bound to an activin type II receptor. In the rightmost lane, excess unlabeled myostatin was included in the binding reaction. (*d*) Binding of myostatin to ActRIIB. All points represent the average of triplicate samples. (*e*) Scatchard analysis of the data shown in *d*. (*f*) Inhibition of myostatin binding to ActRIIB by follistatin (diamonds) and the propeptide (circles). Each experiment was carried out in triplicate, and each curve represents the average of three independent experiments.

on hydroxylapatite, lentil lectin Sepharose, DEAE agarose, and heparin Sepharose. Silver stain analysis of the purified protein preparation revealed the presence of two protein species of 36 and 12.5 kDa (Fig. 1*a*). A variety of data suggested that this purified protein consisted of a noncovalent complex of two propeptide molecules bound to a disulfide-linked C-terminal dimer. First, by Western analysis, the 36- and 12.5-kDa species were immunoreactive with antibodies raised against bacterially expressed fragments of myostatin spanning the propeptide and C-terminal mature region, respectively. Second, in the absence of reducing agents, the C-terminal region had an electrophoretic mobility consistent with that of a dimer. Third, the two species were present in a molar ratio of ≈1:1. And fourth, when the purified protein preparation was passed over a lentil lectin column, the C-terminal dimer was retained on the column and could be eluted with methyl mannose (Fig. 1*b*), even though this portion of the protein contains no potential *N*-linked glycosylation sites; the simplest interpretation of these data is that the C-terminal region bound the lectin indirectly by being present in a tight complex with the propeptide, which does have a glycosylation signal.

Because the C-terminal dimer is known to be the biologically active molecule for other TGF-β family members, we further purified the C-terminal dimer of myostatin away from its propeptide by reverse-phase HPLC. As shown in Fig. 1*a*, the fractions containing the purified C-terminal dimer (fractions 32–34) appeared to be homogeneous. However, the fractions most enriched for the propeptide (fractions 35–37) were contaminated with small amounts of C-terminal dimer (see rightmost lane, Fig. 1*a Center*) and with high molecular weight complexes that most likely represented misfolded proteins.

Most members of the TGF-β superfamily have been shown to signal by binding serine/threonine kinase receptors followed by

Table 1. Muscle weights, mg

Transgenic animals	Pectoralis	Triceps	Quadriceps	Gastrocnemius/plantaris
Male controls (7 mo, n = 10)	100.8 ± 5.4	115.6 ± 5.5	243.8 ± 12.5	168.1 ± 7.6
Dominant-negative Act RIIB (7 mo)				
C5 male founder	148	155	318	252
C11 male founder	227	250	454	338
C33 male founder	158	176	352	244
C42 male founder	196	212	309	269
Female controls (7 mo, n = 10)	68.9 ± 2.7	96.9 ± 3.5	208.3 ± 7.1	140.3 ± 4.3
Dominant-negative Act RIIB (7 mo)				
C2 female founder	104	163	352	263
C4 female founder	103	139	303	194
C27 female founder	135	117	181	256
Male controls (4 mo, n = 12)	98.3 ± 3.3	110.9 ± 2.9	251.7 ± 8.5	169.3 ± 4.7
Follistatin (4 mo)				
F3 male founder	296	494	736	568
F66 male founder	169	263	421	409

All animals (including controls) represent hybrid SJL/C57BL/6 F_0 mice born from injected embryos.

activation of Smad proteins (for review, see refs. 10 and 11). The initial event in triggering the signaling pathway is the binding of the ligand to a type II receptor. To determine whether myostatin is capable of binding any of the known type II receptors for related ligands, we carried out crosslinking studies with radioiodinated myostatin C-terminal dimer on COS-7 cells transfected with expression constructs for either TGF-β, BMP, or activin type II receptors. As shown in Fig. 1*c*, crosslinked complexes of the predicted size (full length receptor bound to myostatin) were detected for cells expressing either Act RIIA or Act RIIB. Because we consistently observed higher levels of binding to Act RIIB than to Act RIIA in both crosslinking and standard receptor-binding assays (data not shown), we focused our receptor-binding studies on Act RIIB. Binding of myostatin to Act RIIB was specific (binding could be competed by excess unlabeled myostatin; Fig. 1*c*) and saturable (Fig. 1*d*), and assuming that all of the myostatin protein was bioactive, we estimated the dissociation constant by Scatchard analysis to be ≈10 nM (Fig. 1*e*). Although this dissociation constant is higher than that reported for other TGF-β family members and their cognate receptors, it is possible that the binding affinity of myostatin for Act RIIB measured in our experiments may not accurately reflect that *in vivo*. For example, it is known in the case of TGF-β that the affinity for the type II receptor is significantly higher in the presence of the appropriate type I receptor (12) and that other molecules are involved in presenting the ligand to the receptor (13, 14).

To determine whether activin type II receptors may be involved in myostatin signaling *in vivo*, we investigated the effect of expressing a dominant-negative form of Act RIIB in mice. For this purpose, we generated a construct in which a truncated form of Act RIIB lacking the kinase domain was placed downstream of a skeletal muscle-specific myosin light chain promoter/enhancer. From pronuclear injections of this construct, we obtained a total of seven founder animals positive for the transgene. All seven showed significant increases in skeletal muscle mass with individual muscles weighing up to 125% more than those of control nontransgenic animals derived from similar injections (Table 1).

Three lines of evidence suggested that the increases in muscle weights in these founder animals resulted from the expression of the transgene. First, analysis of offspring derived from matings of three founder animals (the other four did not generate sufficient numbers of offspring for analysis) with wild-type C57BL/6 mice showed that the increases in muscle weights correlated with the presence of the transgenes (Table 2 and Fig. 2). Second, although muscle weights varied among the different transgenic lines, the magnitude of the increase was highly consistent among animals in any given line for all muscles examined and for both males and females (Table 2). For

Table 2. Muscle weights, mg

Transgenic line	Pectoralis	Triceps	Quadriceps	Gastrocnemius/plantaris
Males				
Controls (n = 50)	104.6 ± 1.5	113.9 ± 1.6	246.2 ± 3.0	167.7 ± 2.1
Dominant-negative Act RIIB				
C5 (n = 11)	153.7 ± 6.0***	177.5 ± 6.0***	322.7 ± 9.3***	247.1 ± 8.1***
C27 (n = 5)	190.4 ± 7.1***	230.8 ± 13.0***	406.8 ± 11.6***	283.8 ± 6.9***
C11 (n = 2)	278.0 ± 18.4*	244.5 ± 4.9**	515.5 ± 7.8**	366.0 ± 21.2*
Propeptide				
B32A (n = 8)	139.9 ± 7.1***	160.6 ± 7.8***	322.5 ± 10.3***	222.6 ± 7.1***
B32B (n = 4)	214.0 ± 19.9**	206.5 ± 6.7***	435.8 ± 15.0***	289.5 ± 8.6***
B32A + B (n = 8)	212.4 ± 8.4***	220.3 ± 6.4***	429.1 ± 11.1***	288.3 ± 8.5***
B53 (n = 8)	215.1 ± 6.4***	229.6 ± 8.1***	413.3 ± 13.2***	293.5 ± 10.5***
Females				
Controls (n = 50)	64.7 ± 1.4	75.7 ± 1.1	164.5 ± 2.0	109.6 ± 1.4
Dominant-negative Act RIIB				
C5 (n = 15)	89.7 ± 2.8***	115.9 ± 4.0***	229.3 ± 6.5***	161.8 ± 4.7***
C27 (n = 5)	117.6 ± 10.9***	138.6 ± 12.3***	314.0 ± 27.7***	207.6 ± 18.3***
C11 (n = 3)	180.3 ± 38.9	208.7 ± 45.7	430.3 ± 72.2*	291.7 ± 48.8*
Propeptide				
B32A (n = 9)	78.8 ± 2.9***	100.1 ± 3.7***	206.0 ± 2.7***	138.9 ± 3.1***
B32B (n = 2)	131.0 ± 18.4	151.5 ± 23.3	315.5 ± 58.7	199.5 ± 24.7
B32A + B (n = 4)	109.3 ± 9.5*	132.8 ± 6.0**	270.8 ± 6.9***	177.0 ± 2.4***
B53 (n = 6)	134.7 ± 7.7***	148.2 ± 12.1***	303.8 ± 18.5***	212.8 ± 12.9***

All animals (including controls) represent 4-month-old offspring of transgenic founders (SJL/C57BL/6) mated with wild-type C57BL/6 mice. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

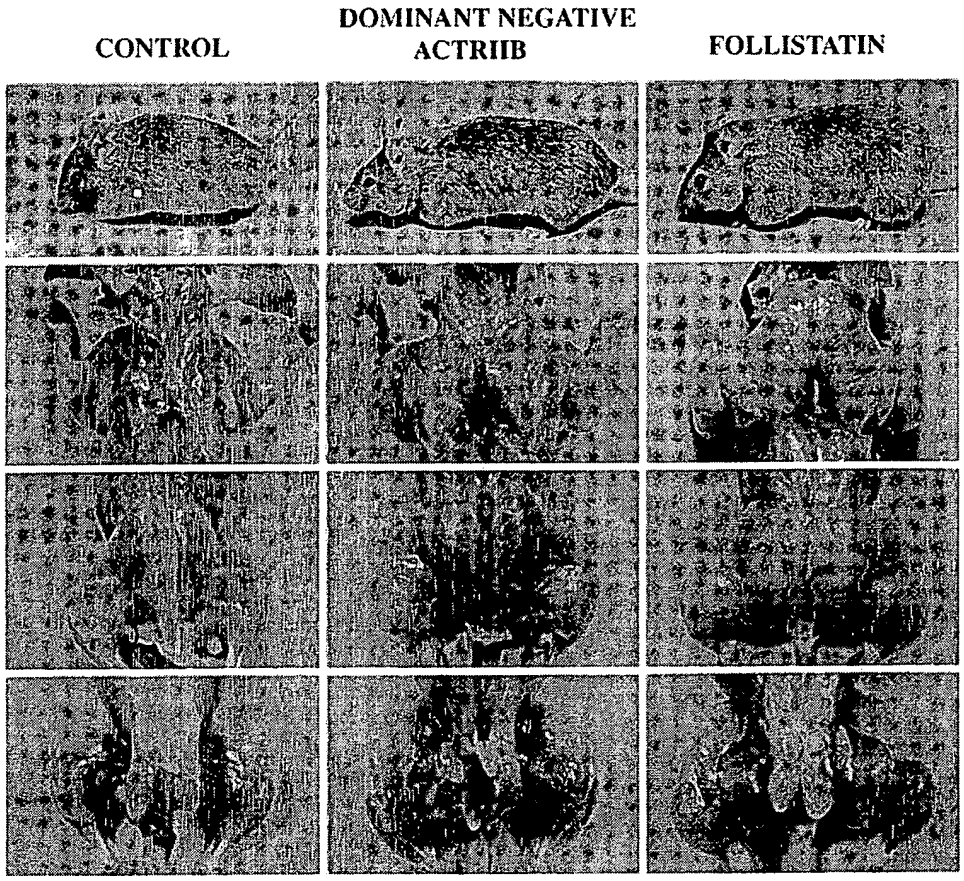

Fig. 2. Increased muscling in mice overexpressing a dominant-negative form of ActRIIB or full length follistatin. A control male nontransgenic mouse, a male transgenic mouse from the C11 line (dominant-negative ActRIIB), and the F3 male founder mouse (follistatin) are shown. Pictures of live mice are shown in the top row, and pictures of animals that had been killed and skinned are shown in the bottom three rows.

example, all muscles of both male and female mice from the C5 line weighed ≈30–60% more than those of control animals, whereas all muscles from C11 mice weighed ≈110–180% more. Third, Northern analysis of RNA samples prepared from transgenic animals showed that the expression of the transgene was restricted to skeletal muscle and that the relative levels of transgene expression (Fig. 3*a*) correlated with the relative magnitude of the increase in muscle weights (Table 2). For example, animals from the C11 line, which had the greatest increases in muscle weights, also had the highest levels of transgene expression.

These data showed that expression of a dominant-negative form of Act RIIB can cause increases in muscle mass similar to those seen in myostatin knockout mice. In myostatin knockout mice, the increase in muscle mass has been shown to result from increases in both fiber number and fiber size (1). To determine whether expression of dominant-negative Act RIIB also causes both hyperplasia and hypertrophy, we analyzed sections of the gastrocnemius and plantaris muscles of animals from the C27 line. Compared with control muscles, the muscles of the C27 animals showed a clear increase in overall cross-sectional area (Fig. 3*b*). This increase in area resulted partially from an increase in fiber number. At the widest point, the gastrocnemius and plantaris muscles had a total of 10,015 ± 1,143 fibers in animals from the C27 line (n = 3) compared with 7,871 ± 364 fibers in control animals (n = 3). However, muscle fiber hypertrophy also contributed to the increase in total area. As shown in Fig. 3*b*, the mean fiber diameter was 51 μm in animals of the C27 line compared with 43 μm in control animals. Hence, the increase in muscle mass appeared to result from an ≈27% increase in the number of fibers and 19% increase in fiber diameter (assuming the fibers to be roughly cylindrical, this increase in diameter would result in an ≈40% increase in cross-sectional area). Except for the increase in fiber number and size, however, the muscles from the transgenic animals looked grossly normal. In particular, there were no obvious signs of degeneration, such as widely varying fiber sizes (note that the standard deviation of fiber sizes was similar between control and transgenic animals) or extensive fibrosis or fat infiltration.

We also used these approaches to explore other possible strategies for inhibiting myostatin. First, we investigated the effect of the myostatin propeptide. In the case of TGF-β, it is known that the C-terminal dimer is held in an inactive latent complex with other proteins, including its propeptide (15), and that the propeptide of TGF-β can have an inhibitory effect on TGF-β activity both *in vitro* (16) and *in vivo* (17). Our observation that the myostatin C-terminal dimer and propeptide copurified raised the possibility that myostatin may normally exist in a similar latent complex and that the myostatin propeptide may have inhibitory activity. Second, we examined the effect of follistatin, which has been shown to be capable of binding and inhibiting the activity of several TGF-β family members. In particular, follistatin can block the activity of GDF-11 (18), which is highly related to myostatin (1, 18–20), and follistatin knockout mice have been shown to have reduced muscle mass at birth (21), which would be consistent with overactivity of myostatin.

We first examined the effect of the propeptide and follistatin *in vitro*. As shown in Fig. 1*f*, both the myostatin propeptide and follistatin were capable of blocking the binding of the C-terminal dimer to Act RIIB. We estimated the K_i of follistatin to be ≈470 pM and that of the propeptide to be at least 50-fold higher. The calculation of the K_i for the propeptide, however, assumes that all

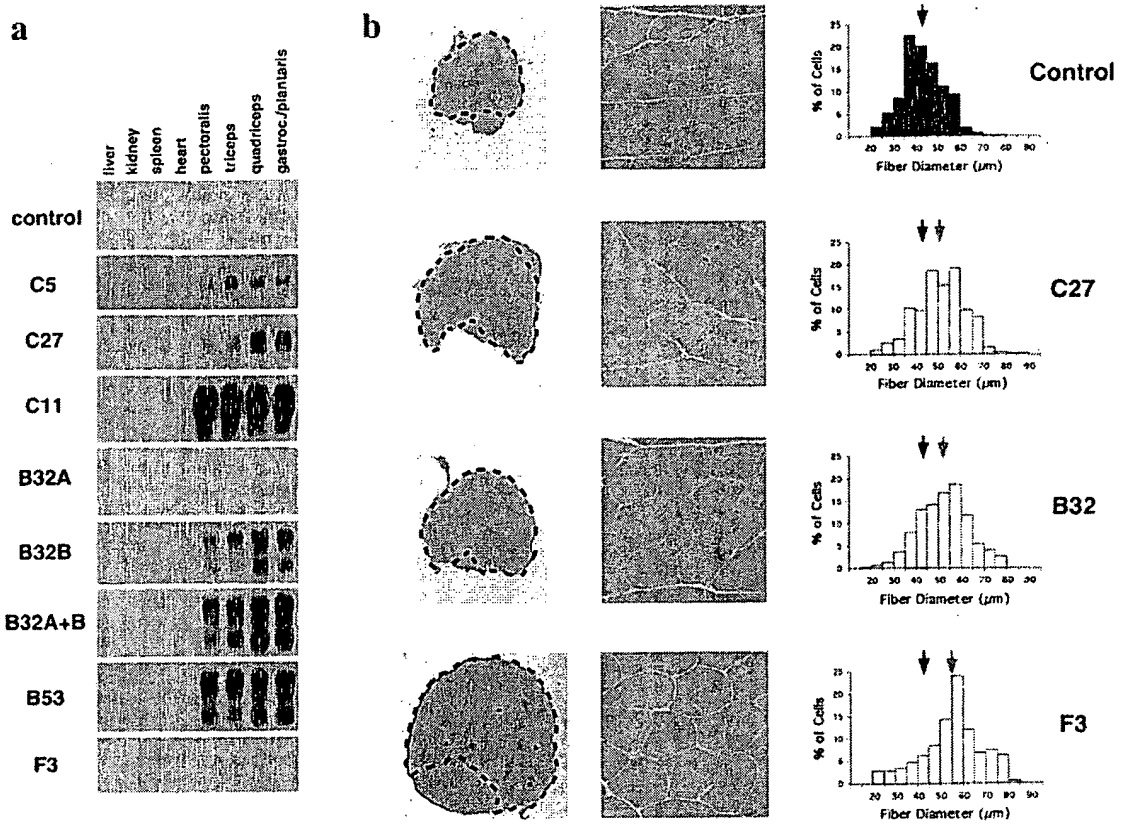

Fig. 3. Analysis of transgenic mice. (*a*) Specific expression of transgenes in skeletal muscle. Northern analysis of RNA samples prepared from female mice was carried out by using simian virus 40 sequences as a probe. On longer exposures of these blots, no expression of the transgenes was observed in liver, kidney, spleen, or heart in any of these lines. (*b*) Muscle analysis. Sections were stained with hematoxylin and eosin. The gastrocnemius and plantaris muscles are outlined (*Left*). *Center* shows higher magnifications of representative areas from the gastrocnemius muscle. *Right* shows distribution of fiber diameters. Each graph represents the composite of 450 fiber measurements from 3 animals (150 per animal), except for the F3 graph, which represents 175 measurements from the F3 founder animal. Standard deviations of fiber sizes were 9, 11, 11, and 13 μm for control, B32, C27, and F3 animals, respectively. Black and gray arrows show the mean fiber diameters for control and transgenic animals, respectively. Note that in each case, the mean fiber diameter was increased in the transgenic animals ($P < 0.001$).

of the protein in the final preparation represented biologically active propeptide and therefore is likely to be an overestimate. As discussed above, our propeptide preparation was contaminated both with small amounts of C-terminal dimer and with misfolded high molecular weight species.

To determine whether these molecules are also capable of blocking myostatin activity *in vivo*, we generated transgenic mice in which the myosin light chain promoter/enhancer was used to drive expression of either the myostatin propeptide or follistatin. From pronuclear injections of the propeptide construct, we obtained three transgenic mouse lines (two of these, B32A and B32B, represented independently segregating transgene insertion sites in one original founder animal) that showed increased muscling. As shown in Table 2, muscle weights of animals from each line were increased by ≈20–110% compared with those of nontransgenic control animals. Northern analysis of RNA samples prepared from representative animals of each of these lines showed that the expression levels of the transgene correlated with the magnitude of the increase in muscle weights (Fig. 3*a*). Specifically, animals from the B32A line, which had only an ≈20–40% increase in muscle mass, had the lowest levels of transgene expression, and animals from the B32B and B53 lines, which had an ≈70–110% increase in muscle mass, had the highest levels of transgene expression. Perhaps significantly, muscle weights in animals that were doubly transgenic for the B32A and B32B insertion sites were similar to those observed in animals transgenic only for the B32B insertion site (Table 2), despite the fact that the doubly transgenic animals appeared to have higher levels of transgene expression (Fig. 3*a*). These findings suggest that the effects seen in the B32B line (and B53 line) were the maximal achievable from overexpressing the propeptide. As in the case of animals expressing the dominant-negative form of Act RIIB, animals expressing the propeptide showed increases in both muscle fiber number and size. Analysis of the gastrocnemius and plantaris muscles from two animals that were doubly transgenic for the B32A and B32B insertion sites showed that fiber numbers were increased by ≈40% (the two animals had 11,940 and 10,420 fibers), and fiber diameters were increased by ≈21% (to 52 μm) compared with control animals.

The most dramatic effects on skeletal muscle were obtained by using the follistatin construct. We obtained two founder animals (F3 and F66) that showed increased muscling (Table 1, Figs. 2 and 3*b*). In one of these animals (F3), muscle weights were increased by 194–327% relative to control animals, resulting from a combination of hyperplasia (66% increase in fiber number to 13,051 in the gastrocnemius/plantaris) and hypertrophy (28% increase in fiber diameter to 55 μm). Although we have not analyzed muscle weights of myostatin knockout mice in a hybrid SJL/C57BL/6 background, the increases in muscle mass observed in the F3 founder animal were significantly greater than the increases we have seen in myostatin null animals in other genetic backgrounds (unpublished

results; see also ref. 1). These results suggest that at least part of the effect of follistatin may result from inhibition of another ligand besides myostatin. Clearly, analysis of additional follistatin transgenic lines will be essential in determining whether other ligands may also be involved in negatively regulating muscle growth.

Discussion

On the basis of the *in vitro* and transgenic mouse data presented here, we propose the following working model for the regulation of myostatin activity. After proteolytic processing, the myostatin C-terminal dimer is maintained in a latent complex with its propeptide and perhaps other proteins as well. Myostatin is also negatively regulated by follistatin, which binds the C-terminal dimer and inhibits its ability to bind to receptors. Release of the C-terminal dimer from these inhibitory proteins by unknown mechanisms allows myostatin to signal through activin type II receptors. By analogy with other family members, we presume that activation of these receptors then leads to activation of a type I receptor and Smad proteins.

Our overall model for myostatin regulation and signaling is consistent not only with the data presented here but also with other genetic data. As discussed earlier, follistatin knockout mice have been shown to have reduced muscle mass at birth (21), which is what one might expect for uninhibited myostatin activity. A similar muscle phenotype has been reported for mice lacking ski (22), which has been shown to inhibit the activity of Smad2 and 3 (23–27), and the opposite phenotype, namely excess skeletal muscle, has been observed in mice overexpressing ski (28). On the basis of our findings, an appealing hypothesis is that these observed phenotypes reflect the overactivity and underactivity, respectively, of myostatin in these mice.

Although all of the *in vitro* and genetic data are consistent with the overall model that we have put forth here, these data would also be consistent with alternative models involving other receptors and ligands. For example, we do not know the mechanism by which the truncated form of Act RIIB enhances muscle growth in our transgenic mice. It is possible that the truncated receptor is not acting to block signaling in the target cell but is rather merely acting as a sink to deplete extracellular concentrations of myostatin. It is also possible that the truncated receptor is blocking signaling of other ligands besides myostatin. In other species, it has been shown that dominant-negative forms of type II activin receptors can block signaling of a variety of different TGF-β-related ligands (29–32). Similarly, our data do not show definitively that follistatin is blocking myostatin activity *in vivo* to promote muscle growth. In this regard, the extraordinary degree of muscling seen in one of the follistatin-expressing founder animals suggests that other follistatin-sensitive ligands may be involved in regulating muscle growth. One obvious candidate is GDF-11, which is highly related to myostatin (1, 18–20) and also expressed in skeletal muscle (unpublished results). Moreover, it is known that GDF-11 activity in *Xenopus* can be blocked by follistatin (18). Other candidate ligands would also include the activins, which have been shown to be capable of inhibiting muscle cell differentiation *in vitro* (33).

To date, however, myostatin is the only secreted protein that has been demonstrated to play a negative role in regulating muscle mass *in vivo*. Although additional experiments will be required to prove aspects of this overall model and to identify the other signaling components, our data suggest that myostatin antagonists, such as follistatin and the myostatin propeptide, or activin type II receptor antagonists may be effective muscle-enhancing agents for both human and agricultural applications.

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NiceProt View of Swiss-Prot: P27037

Entry information

Entry name	**AVR2_HUMAN**
Primary accession number	**P27037**
Secondary accession number	Q92474
Entered in Swiss-Prot in	Release 23, August 1992
Sequence was last modified in	Release 23, August 1992
Annotations were last modified in	Release 43, March 2004

Name and origin of the protein

Protein name	**Activin receptor type II [Precursor]**
Synonyms	**EC 2.7.1.37** **ACTR-II** **ACTRIIA**
Gene name	**ACVR2**
From	Homo sapiens (Human) [TaxID: 9606]
Taxonomy	Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.

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[4] SEQUENCE FROM NUCLEIC ACID.
Iimura T., Oida S.;
Submitted (NOV-1994) to the EMBL/GenBank/DDBJ databases.

Comments

- ***FUNCTION***: Receptor for activin A, activin B and inhibin A. Involved in transmembrane signaling.
- ***CATALYTIC ACTIVITY***: ATP + a protein = ADP + a phosphoprotein.

- ***SUBUNIT***: Interacts with AIP1. Part of a complex consisting of AIP1, ACVR2, ACVR1B and MADH3 *(By similarity)*.
- ***SUBCELLULAR LOCATION***: Type I membrane protein.
- ***SIMILARITY***: Belongs to the Ser/Thr protein kinase family. TGFB receptor subfamily.

Copyright

Cross-references

EMBL	X63128; CAA44839.1; -. X62381; CAA44245.1; -. M93415; AAA35504.1; -. D31770; BAA06548.1; -.
PIR	JQ1486; JQ1486.
HSSP	P27038; 1BTE.
Genew	HGNC:173; ACVR2.
CleanEx	HGNC:173; ACVR2.
GeneCards	ACVR2.
GeneLynx	ACVR2; Homo sapiens.
GenAtlas	ACVR2.
MIM	102581 .
GO	GO:0005887;Cellular component: integral to plasma membrane *(traceable author statement)*. GO:0004675;Molecular function: transmembrane receptor protein serine/threonine kinase activity *(traceable author statement)*. GO:0007178;Biological process: transmembrane receptor protein serine/threonine kinase signaling pathway *(traceable author statement)*.
InterPro	IPR000472; Activin_receptor. IPR000333; Actn_receptorII. IPR000719; Prot_kinase. IPR008271; Ser_thr_pkin_AS.
Pfam	PF01064; Activin_recp; 1. PF00069; pkinase; 1. Pfam graphical view of domain structure.
PRINTS	PR00653; ACTIVIN2R.
ProDom	PD000001; Prot_kinase; 1. [Domain structure / List of seq. sharing at least 1 domain]
PROSITE	PS00107; PROTEIN_KINASE_ATP; FALSE_NEG. PS00108; PROTEIN_KINASE_ST; 1. PS50011; PROTEIN_KINASE_DOM; 1. PROSITE graphical view of domain structure.
Implicit links to	SOURCE; Ensembl; HOVERGEN; BLOCKS; ProtoNet; ProtoMap; PRESAGE; DIP; ModBase; SMR; SWISS-2DPAGE; UniRef.

Keywords

Receptor; Transferase; Serine/threonine-protein kinase; ATP-binding; Transmembrane; Glycoprotein; Signal.

Features

Key	From	To	Length	Description
SIGNAL	1	19	19	*Potential.*
CHAIN	20	513	494	Activin receptor type II.
DOMAIN	20	135	116	Extracellular (*Potential*).
TRANSMEM	136	161	26	*Potential.*
DOMAIN	162	513	352	Cytoplasmic (*Potential*).
DOMAIN	192	485	294	Protein kinase.
NP_BIND	198	206	9	ATP (*By similarity*).
BINDING	219	219		ATP (*By similarity*).
ACT_SITE	322	322		*By similarity.*
DISULFID	30	60		*By similarity.*
DISULFID	50	78		*By similarity.*
DISULFID	85	104		*By similarity.*
DISULFID	91	103		*By similarity.*
DISULFID	105	110		*By similarity.*
CARBOHYD	43	43		N-linked (GlcNAc...) (*Potential*).
CARBOHYD	66	66		N-linked (GlcNAc...) (*Potential*).
CONFLICT	13	13		L -> V (in Ref. 4).
CONFLICT	204	206		GCV -> PSL (in Ref. 4).
CONFLICT	348	348		E -> V (in Ref. 4).

Sequence information

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Molecular weight: **57847 Da** [This is the MW of the unprocessed precursor]
CRC64: **A89822E880979618** [This is a checksum on the sequence]

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       190        200        210        220        230        240
         |          |          |          |          |          |
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         |          |          |          |          |          |
AYLHEDIPGL KDGHKPAISH RDIKSKNVLL KNNLTACIAD FGLALKFEAG KSAGDTHGQV

       370        380        390        400        410        420
         |          |          |          |          |          |
GTRRYMAPEV LEGAINFQRD AFLRIDMYAM GLVLWELASR CTAADGPVDE YMLPFEEEIG

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         |          |          |          |          |          |
QHPSLEDMQE VVVHKKKRPV LRDYWQKHAG MAMLCETIEE CWDHDAEARL SAGCVGERIT

       490        500        510
         |          |          |
QMQRLTNIIT TEDIVTVVTM VTNVDFPPKE SSL
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Inactivation of Activin-Dependent Transcription by Kinase-Deficient Activin Receptors*

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ABSTRACT

Activin, a member of the transforming growth factor-β superfamily, binds to two classes of cell surface receptors. These receptors, designated type I and type II, are structurally related members of transmembrane serine kinase superfamily. Antibodies specific for either type I or type II activin receptor can coprecipitate complexes containing both affinity-labeled receptors from activin-responsive cells. Two type I receptors show cell-specific expression and associate with the ligand-binding, type II receptors. To investigate the roles of the cytoplasmic receptor domains in signaling through a heteromeric ligand receptor complex, we have made kinase-deficient activin receptors and correlated their losses in kinase activity with inhibitory effects on an activin-dependent transcriptional response in activin-responsive cell lines. Wild-type activin type II receptors phosphorylate activin type I receptors in transfected COS cells. In contrast, kinase-deficient activin type II receptors fail to phosphorylate type I receptors in transfected COS cells and act as dominant negative mutants to block activin-induced transcriptional activity in both Chinese hamster ovary and K562 (human erythroleukemia) cells. Kinase-deficient activin type IB receptors also block activin-induced transcriptional activity in both Chinese hamster ovary and K562 cells, whereas kinase-deficient activin type I receptors have no effect in either cell line. These results indicate that kinase activities of both type II and type I receptors are required for activin signaling, and that the two type I receptors, which are expressed in a tissue-specific manner, are functionally distinct. (*Endocrinology* **136:** 5493–5503, 1995)

ACTIVINS belong to the transforming growth factor-β (TGFβ) superfamily of growth and differentiation factors that also includes Mullerian inhibiting substance, multiple bone morphogenetic proteins (BMPs), the *Drosophilia* decapentapegic and Vg60A gene products, *Xenopus* Vg1, and dorsalin and nodal, which play important roles in early development (1–4). After their initial characterization as stimulators of FSH production from the anterior pituitary (1, 2), activins were found to be produced by reproductive and other tissues where they exert important endocrine, paracrine, and autocrine actions to regulate cell proliferation, development, and differentiated functions. The biological actions of activins include erythroid differentiation, nerve cell survival, modulation of pituitary hormone secretion, regulation of steroidogenesis and gametogenesis, and induction of mesoderm in *Xenopus laevis* (for review, see Ref. 3). Differential association of two structurally related polypeptide chains, βA and βB, results in three homo- and heterodimeric forms of activins: activin A (βAβA), activin B (βBβB), and activin AB (βAβB) (1–4). Recently, new two activin β-subunits have been reported; βC from human liver and βD from *Xenopus* (5, 6). The biological activities of these putative activins remain to be determined. Affinity labeling and chemical cross-linking of activin-responsive cells reveal two major activin receptor complexes: a type I complex of approximately 60–70 kilodaltons (kDa) and a type II complex of approximately 80–90 kDa (7). Several complementary DNAs (cDNAs) of both types of receptors have been cloned and encode proteins comprising a small extracellular domain, a single transmembrane domain, and a cytoplasmic component with a protein-serine kinase domain (8–10). After our initial identification of the type II activin receptor as the first vertebrate receptor serine kinase (RSK) (7), multiple members of this novel RSK superfamily have been identified, including a second type of activin type II receptor (ActRIIB) (11, 12), the type II receptor for TGFβ (13), and several type I receptors for activin, TGFβ, and other TGFβ superfamily members (14). The two known type II activin receptors (ActRII and ActRIIB) show high affinity activin binding (K_d = 200–700 pM) when expressed in COS cells (7, 8, 11, 12). In contrast, and similar to that observed for TGFβ and its receptors, type I activin receptors (ActRI and ActRIB) do not bind activin without the coexpression of type II receptors (8–10). Transfected ActRIs form a detectable complex with activin and ActRIIs (8, 10). Formation of a complex of type I and type II receptors is required to mediate biological responses to activin, and type I receptors appear to specify some of the responses (15).

The signaling mechanism of growth factors through re-

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ceptor tyrosine kinases (RTKs) has been well characterized (16–18). After ligand binding, dimerizaton of homo- or heteromeric RTKs induces receptor autophosphorylation, which then triggers downstream signaling. Intracellular signaling molecules activated by RTKs include phosphoinositide-3-kinase, S6 kinase, mitogen-activated protein kinases, phospholipase C-γ, and the JAK/STAT family members (16–18). In contrast, the signaling mechanism through RSKs is not fully understood.

To understand the roles of the cytoplasmic kinase domains and the kinase activities of activin receptors in signaling through a heteromeric ligand receptor complex, we have made mutant receptors in which the kinase domain has been deleted or mutated. We analyzed the complex formation and dominant-negative effects of kinase-deficient receptors. We provide evidence that the kinase domain and kinase activities of type II receptors are not required for binding to activin or association with type I receptors, but are required for signaling. Furthermore, we report that kinase-deficient ActRIBs act as dominant negative mutants to block an activin-dependent transcriptional response in activin-responsive cells, whereas kinase-deficient ActRIs have no effect.

Materials and Methods

Materials

Recombinant human activin A was kindly provided by J. Mather (Genentech, South San Francisco, CA).

Construction of mutant receptors and transfection

All cDNAs were subcloned into pcDNA1 unless otherwise indicated. cDNAs for mouse ActRII, mouse ActRIIB, ActRIIB(K217R), and rat ActRI were described previously (8, 19). Human ActRIB(ALK4) cDNA in pcDNA1 was obtained from Dr. K. Miyazono. ActRI(K235R), a point mutant of rat ActRI, was made by mutation of a.lysine residue 235 to arginine using polymerase chain reaction (PCR) mutagenesis. ΔActRII(HA), a deletion mutant of ActRII that encodes 174 amino acids (aa) of ActRII and the 9-aa influenza virus hemagglutinin (HA) epitope at the C-terminus (20), was constructed using mouse ActRII as a PCR template with 3 primers, as described below. The 5'-primer was the T7 primer (5'-AATACGACTCACTATAG-3'). The patch primer (5'-GAGGCTAGCATAATCAGGAACATCATACTGCAATGGCTTCAACCC-3') coded for the complementary sequences of GLKPLQ (aa 169–174 of ActRII) and YDVPDYASL (HA epitope sequence). The 3'-primer (5'-GGCCCTCTAGAGCTAGAGGCTAGCATAATC-3') consisted of 15 nucleotides identical to the patch primer, stop codon, and unique *Xba*I site. The 5'- and 3'-primers were used at 100 pmol each, whereas the patch primer was used at 1 pmol/PCR reaction. The amplified ΔActRII(HA) product was digested with *Xba*I, gel purified, subcloned into pcDNA1, and sequenced to verify the PCR amplification reaction. ΔActRIB(*myc*), a deletion mutant of ActRIB that encodes 185 aa of ActRIB and 5 consecutive *myc* epitopes (MEQKLISEEDLNE) at the C-terminus, was constructed as follows. Sequences encoding *myc* epitope were PCR amplified using CS2+MT(*myc* tag) plasmid (kindly provided by Dr. H. Weintraub, Fred Hutchinson Cancer Research Center, Seattle, WA), and ligated to *Acc*I-*Xho*I-digested ActRIB cDNA in pcDNA1. ΔActRI(*myc*), a deletion mutant of ActRI that encodes 163 aa of ActRI and 5 consecutive *myc* epitopes, was constructed by ligating the PCR-amplified *myc* sequence to *Bst*EII-*Xho*I-digested ActRI cDNA in pcDNA1. All constructs were verified by nucleotide sequencing. DNAs were transfected into COS-M6 cells by diethylaminoethyl-dextran methods as previously described (8). For cotransfection of two receptor cDNAs, type I receptor cDNAs (ActRI or IB cDNAs, either wild type or mutated) and type II receptor cDNAs (ActRII or IIB cDNAs, either wild type or mutated) were transfected at a DNA ratio of 3 type I to 1 type II. This ratio is optimal for the visualization of an affinity-labeled type I receptor band in COS cells, which is comparable to endogenous receptor complexes seen in activin-responsive cells (7, 21).

Binding and chemical cross-linking

Recombinant activin A was iodinated using the chloramine-T oxidation method, as previously described (22), to specific activities of approximately 50–100 μCi/μg. RRAs and chemical cross-linking to cells with disuccinimidyl suberate (DSS) were performed as described previously (8).

Antibodies

Peptides predicted from the carboxy-terminal sequences of ActRI (LRIKKTLTKIDNSLDKLKTDC; aa 469–489) and ActRIB (TLSQLSVQEDVKI; aa 470–482), with the substitution of tyrosine for cysteine at the carboxy-terminal of ActRI and the addition of tyrosine-glycine at the amino-terminus of Act RIB, were coupled to keyhole limpet hemocyanin via bisdiazotized benzidine and used as immunogen, as described for inhibin-α (23). Antisera were raised in rabbits using complete Freund's adjuvant (initial injection) or incomplete Freund's adjuvant (booster injections). Antiserum from either of two rabbits (ARI-5534 or ARI-5536) was used for immunoprecipitation of full-length or point-mutated ActRI, antiserum from rabbit 5626 (ARIB-5626) or affinity-purified ActRIB antibody 277–143-C (3.78 ml/ml serum equivalents) was used for immunoprecipitation of ActRIB, antiserum for ActRII was described previously (19), and the monoclonal antibody 12CA5 for HA epitope and monoclonal antibody 9E10 for *myc* epitope were purchased from Berkeley Antibody Co. (Babco, Berkeley, CA) and Oncogene Science (Manhasset, NY), respectively.

Immunoprecipitation

Affinity-labeled receptors, solubilized in 1% Triton X-100, were diluted to 500 μl in lysis buffer [20 mM Tris-Cl (pH 7.8), 2 mM EDTA, 300 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 1% aprotinin, and 0.2 mM phenylmethylsulfonylfluoride] and immunoprecipitated with antiserum as follows: 6 μl ActRI antiserum ARI-5536, 12 μl ActRIB antiserum ARIB-5626, 4 μl ActRII antiserum ARII-5283, 10 μl HA antibody 12CA5 for ΔActRII(HA), and 10 μl *myc* antibody 9E10 for ΔActRIB(*myc*) or ΔActRIB(*myc*). Immune complexes were collected with 60 μl of a 25% slurry of protein A-Sepharose (Pharmacia LKB Biotechnology, Piscataway, NJ) for activin receptor and HA antibodies, or protein G-Sepharose (Sigma) for *myc* antibody. For deglycosylation, samples were treated with 250 U peptide *N*-glycosidase F (New England Biolabs, Beverly, MA) for 1 h after immunoprecipitation. Samples were analyzed using 7.5% acrylamide-SDS-gel electrophoresis (SDS-PAGE). All methods were previously described in detail for ActRII (19). Where indicated, 50 μg/ml of the appropriate peptide competitor were added to samples before incubation with antibody.

Cell surface biotinylation

Confluent COS cells in 3.5-cm dishes, transfected with various combinations of wild-type and mutant receptors, were surface labeled with 300 μg/ml D-biotinyl-ε-amidocaproic acid *N*-hydroxysuccinimide ester (biotin-CNHS-ester, Boehringer Mannheim, Indianapolis, IN) as previously described (24). After biotinylation, labeled cells were solubilized, and supernatants were precleared with *Staphylococcus aureus* (Pansorbin, Calbiochem, La Jolla, CA) that was preadsorbed with normal rabbit serum, and immunoprecipitated with 3.5 μl ActRI antiserum ARI-5536. The immunoprecipitates were run on 7.5% acrylamide-SDS-PAGE and transferred to nitrocellulose sheets by electroblotting, and biotin-labeled proteins were detected with horseradish peroxidase-conjugated streptavidin using a chemiluminescent system (ECL; Amersham Corp., Arlington Heights, IL) following the manufacturer's instructions. For the cell surface biotinylation of ΔActRIB(*myc*) and ΔActRI(*myc*) in transfected K562 cells, 6×10^6 transfected cells were lysed as described above and immunoprecipitated with 10 μl *myc* monoclonal antibody

9E10, and biotin-labeled proteins were detected as described above, except that 12% acrylamide-SDS-PAGE was used.

[^{32}P]Orthophosphate labeling and phosphoamino acid analysis

Metabolic labeling with [^{32}P]orthophosphate was performed according to our previous procedure with slight modifications (19). In brief, transfected COS cells in six-well dishes were labeled with 1 mCi/ml [^{32}P]orthophosphate in phosphate-free Dulbecco's Modified Eagle's Medium (DMEM) without serum for 3.5 h, and ActRI or ActRIB protein was immunoprecipitated with 6 μl ARI-5536 or 6 μl affinity-purified ARIB, respectively. Samples were run on 7.5% acrylamide-SDS-PAGE without deglycosylation, and the gels were blotted to Immobilon (Millipore Corp., Bedford, MA) followed by autoradiography. Two-dimensional phosphoamino acid analysis was performed after excising the ^{32}P-labeled bands from the filters as described previously (19, 25).

Transcriptional assay for activin receptors

p3TP-Lux, which contains three copies of a 12-*O*-tetraphorbol acetate-responsive element and ~100 base pairs of the promoter of the human plasminogen activator inhibitor-1 linked to the luciferase reporter gene, was obtained from Dr. J. Massagué (8, 13, 26). Chinese hamster ovary (CHO) cells, grown in DMEM containing 10% fetal calf serum (FCS), were plated at a density of 4.0×10^5 cells/well in six-well dishes. After 24 h, cells were transfected with 2.5 μg p3TP-Lux and various amounts of ΔActRII(HA), ΔActRIB(*myc*), or ΔActRI(*myc*) cDNA by DEAE-dextran method, as previously described (8). For the internal control of transfection efficiency, 2.5 μg of a β-galactosidase expression plasmid [Rous sarcoma virus (RSV)-βgal or cytomegalovirus (CMV)-βgal] were also cotransfected. The total amount of DNAs transfected was adjusted by cotransfecting the parental plasmid pcDNA1 or pcDNA3 (Invitrogen, San Diego, CA). After 24 h of transfection, cells were stimulated with or without 2 nM activin A in DMEM containing 0.2% FCS for 24 h. K562 cells were grown in RPMI 1640 containing 10% FCS (complete medium), washed in HEPES-buffered saline (HDB), and resuspended at 4×10^7 cells/ml in HDB. Two $\times 10^7$ cells in 500 μl were transfected by electroporation (Bio-Rad Gene Pulser, Bio-Rad Laboratories, Richmond, CA; 960 μF, 0.22 kV) with 20 μg p3TP-Lux, 10 μg CMV-βgal, and various amounts of receptor cDNA and parental vector cDNA to total 20 μg. Cells were resuspended in 10 ml complete medium and cultured in 100-mm dishes for 24 h. Transfected cells were then washed in HDB and resuspended in 6 ml RPMI 1640 containing 0.5% FCS. Cells were plated at 1 ml/well in 12-well dishes and treated with or without 800 pM activin A for 12 h. The luciferase activity of each lysate was measured and normalized to the β-galactosidase activity (8).

Results

Association of activin type I and type II receptors in activin-responsive cells

To study the properties of type I receptors in intact cells, we generated polyclonal antisera against peptides predicted from the carboxy-terminal 21 aa of rat ActRI (8) and the carboxy-terminal 13 aa of human ActRIB (15). These regions are conserved among different mammalian species (8, 15, 27). We used these antisera (ARI-5536 or ARIB-5626) in conjunction with an antiserum against the carboxy-terminal 13 aa of Act RII (ARII-5283) to study the association of type I and type II receptors in activin-responsive cells. [^{125}I]Activin A-labeled type I and type II complexes were coprecipitated with either ActRII antiserum ARII-5283 or ActRIB antiserum ARIB-5626, but not with ActRI antiserum ARI-5536 in CHO cells (Fig. 1A). In the human erythroleukemic cell line, K562, activin A affinity-labeled complexes were clearly coprecipitated with either ARII-5283 or ARIB-5626, but more weakly

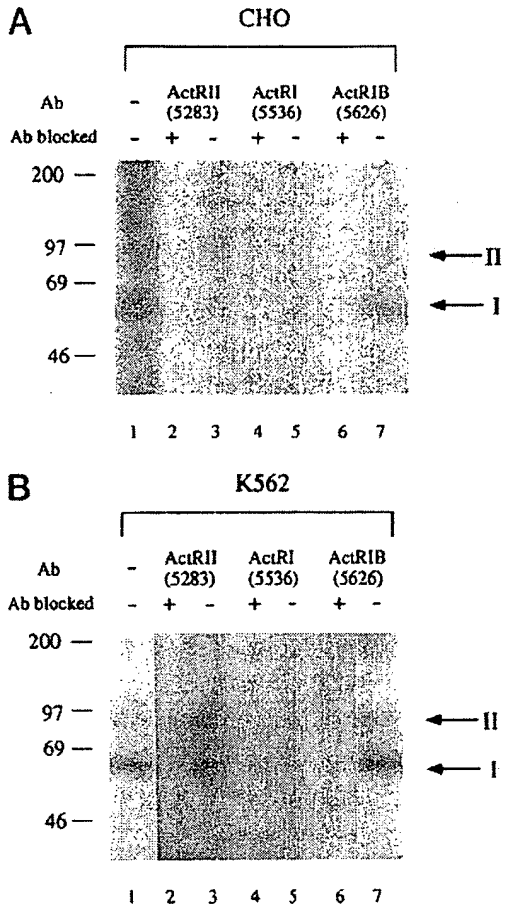


FIG. 1. Association of activin receptors in activin-responsive cells. Activin-responsive CHO (A) and K562 (B) cells were bound with 2 nM [^{125}I]activin A and cross-linked with DSS. The detergent-solubilized fraction was immunoprecipitated with ActRII antibody 5283 (lanes 2 and 3), ActRI antibody 5536 (lanes 4 and 5), or ActRIB antibody 5626 (lanes 6 and 7), either unblocked (lanes 3, 5, and 7) or blocked (lanes 2, 4, and 6) with 50 μg/ml of the cognate peptide immunogens, and resolved by SDS-PAGE under reducing conditions. In lane 1, total lysates were analyzed. M_r markers, in kilodaltons, are indicated on the *left*; the positions of type I and type II receptors are indicated on the *right*.

with ARI-5536 (Fig. 1B). Immunoprecipitation of these affinity-labeled ActRs was completely abolished by coincubation of the antisera with cognate peptide immunogen (Fig. 1, a and b, lanes 2, 4, and 6). These data indicate that ActRIB is a component of the activin receptor complexes in these two cell types. On the other hand, ActRI is a component of the activin receptor complex in K562 cells, but not in CHO cells, although both cells abundantly express ActRI messenger RNA (8). Thus, the association of different activin type I receptors with ligand-binding type II receptors is cell specific.

Complex formation by point-mutated kinase-defective type I and type II receptors

We next studied receptor-ligand complex formation using the point-mutated receptors ActRIIB(K217R) and ActRI(K235R), in which lysine residues have been replaced

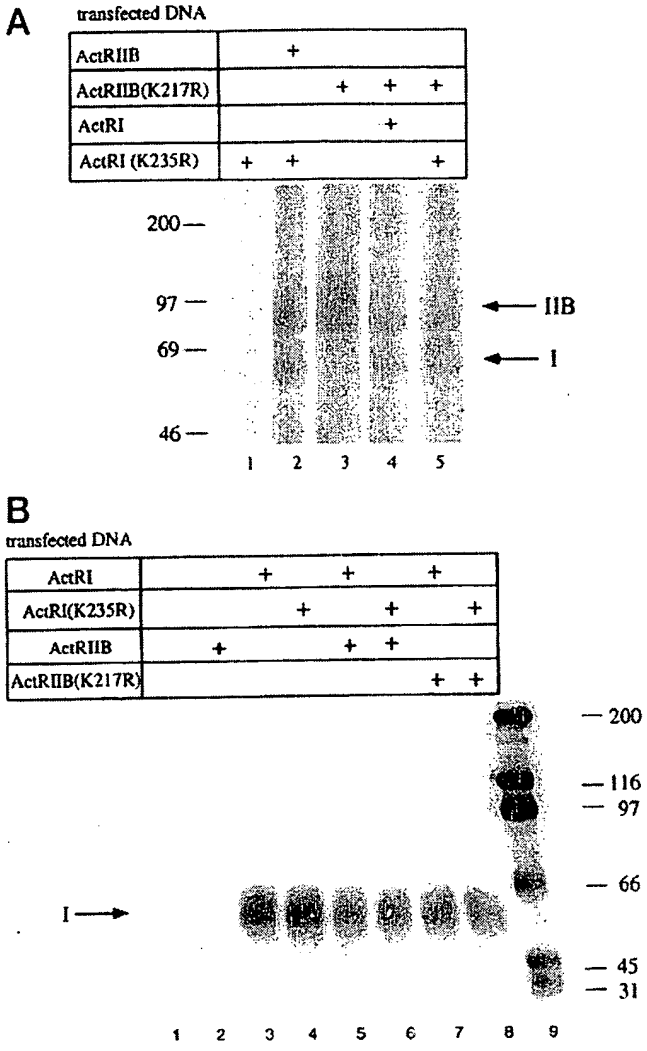


FIG. 2. Complex formation of point-mutated activin receptors. A, COS cells transfected with various combinations of wild-type and point-mutated activin receptors were bound with 600 pM [^{125}I]activin A and cross-linked with DSS. The detergent-solubilized fractions were analyzed by SDS-PAGE under reducing conditions. The transfected DNAs are indicated. M_r markers (kilodaltons) and the positions of ActRI and ActRIIB are indicated on the *left* and *right*, respectively. B, Cell surface expression of ActRI in transfected COS cells. As indicated at the *top*, wild-type or point-mutated ActRI was transfected either alone or with ActRIIB (either wild type or point mutated). Cell surface proteins were labeled by biotin as described in *Materials and Methods*. Biotin-labeled ActRI was immunoprecipitated with antibody 5536, resolved by SDS-PAGE, transferred to nitrocellulose, and visualized using the ECL system (Amersham). M_r markers, in kilodaltons, are indicated on the *right*; the position of ActRI is indicated on the *left*. Lane 9 contains the biotin-labeled mol wt markers (Bio-Rad).

by arginine. These lysine residues in kinase subdomain II are completely conserved within the entire family of protein kinases and are considered critical for catalytic activity (28). Complex formation was analyzed by affinity cross-linking using [^{125}I]activin A. Similar to what was found for wild-type ActRI, COS cells transfected with ActRI(K235R) alone did not bind [^{125}I]activin A (Fig. 2A, lane 1). However, [^{125}I]activin-labeled type I receptor complexes were observed when cells were cotransfected with ActRI(K235R) and either wild-type or point-mutated kinase-deficient ActRIIB (Fig. 2A, lanes 2 and 5). COS cells transfected with ActRIIB(K217R) formed complexes with [^{125}I]activin that were indistinguishable from those observed for the native ActRIIB (Fig. 2A, lane 3). Additionally, ActRIIB(K217R) formed complexes either with wild-type or point-mutated ActRI (Fig. 2A, lanes 4 and 5). ActRI, either wild type or point mutated, decreased the binding of ActRIIB(K217R) to activin (Fig. 2A, lanes 3–5). These data indicate that the kinase activities of activin receptors are not critical for heteromeric receptor complex formation and inhibition of type II receptor activin binding by type I receptor.

We examined whether cell surface expression of ActRI is dependent on the presence of type II receptors or is affected by point mutations in the kinase domain of either type I or type II receptors. Cell surface proteins of COS cells transfected with ActRI were labeled with biotin, and the detergent-soluble cell lysate was precipitated using ActRI antiserum ARI-5536. Protein bands of approximately 58 kDa were visualized with horseradish peroxidase-conjugated streptavidin in cells transfected with either ActRI or ActRI (K235R), consistent with the predicted size of glycosylated ActRI (Fig. 2B, lanes 3 and 4) (8). No reaction product was seen in either mock-transfected cells or cells transfected with ActRIIB alone (Fig. 2B, lanes 1 and 2), confirming the specificity of the ActRI antiserum. Additionally, immunoprecipitation of biotinylated ActRI was abolished by coincubation of the ActRI antiserum with the cognate peptide immunogen. Both ActRI and ActRI (K235R) were expressed on the cell surface, either alone or cotransfected with ActRIIB. These results together with binding data support the idea that ActRI does not independently bind activin even when exposed on the cell surface.

Activin type I receptors undergo ActRIIB-dependent phosphorylation in transfected COS cells

We and others have reported that type II activin receptors are phosphoproteins (19, 29). In transfected COS cells, the phosphorylation of ActRII occurs primarily on serine residues and arises, to some extent, from autophosphorylation (19). To address whether auto- and/or transphosphorylation between the two receptors might be the primary effects of receptor interaction, we have transfected COS cells with various combinations of wild-type or point-mutated kinase-defective type I and type II receptors to study their phosphorylation. Because the cell surface expression and protein levels of activin type I receptors in transfected cells are comparable regardless of type II receptor expression and receptor kinase domain mutations (Fig. 2B, lanes 5–8, and not shown), the level of phosphorylation of type I receptors can be studied *in situ*. As shown in Fig. 3A, ActRI was weakly phosphorylated in COS cells transfected either alone or with ActRIIB(K217R) (Fig. 3A, lanes 1–4). In contrast, ActRI was highly phosphorylated when cotransfected with wild-type ActRIIB (Fig. 3A, lane 6). A similar degree of phosphorylation was seen when ActRI(K235R), rather than wild-type ActRI, was cotransfected with wild-type ActRIIB (Fig. 3A, lane 8). Thus, ActRI phosphorylation is mainly ActRIIB de-

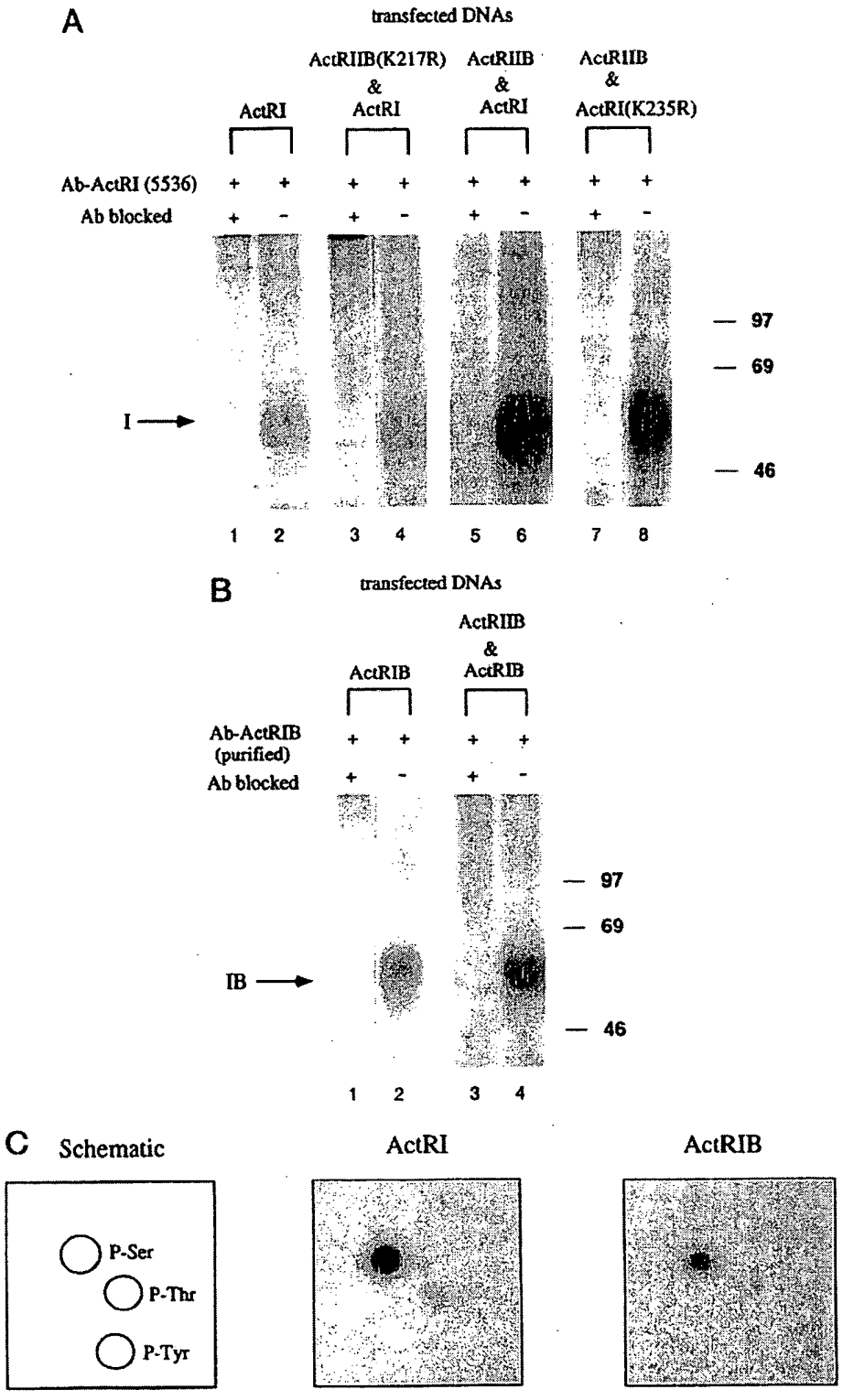


FIG. 3. *In situ* phosphorylation of activin type I receptors. A, COS cells were transfected with ActRI (lanes 1 and 2), ActRIIB(K217R) and ActRI (lanes 3 and 4), ActRIIB and ActRI (lanes 5 and 6), or ActRIIB and ActRI(K235R) (lanes 7 and 8). Cells were metabolically labeled with [^{32}P]orthophosphate, immunoprecipitated with ActRI antibody either unblocked (lanes 2, 4, 6, and 8) or blocked (lanes 1, 3, 5, and 7) with 50 μg/ml of the cognate peptide immunogen, resolved by SDS-PAGE, transferred to Immobilon, and autoradiographed. M_r markers, in kilodaltons, are indicated on the *right*; the position of ActRI is indicated on the *left*. B, COS cells were transfected with either ActRIB (lanes 1 and 2), or ActRIIB and ActRIB (lanes 3 and 4). Cells were metabolically labeled with [^{32}P]orthophosphate, immunoprecipitated with affinity-purified ActRIB antibody either unblocked (lanes 2 and 4) or blocked (lanes 1 and 3) with 50 μg/ml of the cognate peptide immunogen, resolved by SDS-PAGE, transferred to Immobilon, and autoradiographed. M_r markers, in kilodaltons, are indicated on the *right*; the position of ActRIB is indicated on the *left*. C, COS cells were cotransfected with ActRIIB and ActRI, or ActRIIB and ActRIB, and metabolically labeled with [^{32}P]orthophosphate. Solubilized cell lysates were immunoprecipitated with ActRI or ActRIB antibody, respectively, resolved on SDS-PAGE, and transferred to Immobilon. The activin type I receptor bands were excised from the filter, hydrolyzed in 6 N HCl, and subjected to two-dimensional thin layer electrophoresis as described previously (19). The positions of the phosphoamino acids are schematically indicated in the *left panel*. The autoradiograms of ^{32}P-labeled ActRI and ActRIB are shown in the *middle and right panels*, respectively.

pendent, and autophosphorylation plays only a small role. Similarly, ActRIIB-dependent ActRIB phosphorylation was observed when ActRIIB and ActRIB were cotransfected in COS cells (Fig. 3B). Cotransfection of activin type I receptors (ActRI and ActRIB) with ActRIIB gave results similar to cotransfection of ActRIs with ActRII, although ActRII expression was generally less than that of ActRIIB in all transfection experiments (not shown). Contrary to the significant increase in phosphorylation of activin type I receptors upon coexpression of ActRIIB, the apparent level of ActRIIB phosphorylation was

not significantly changed by cotransfection with activin type I receptors, even upon activin treatment (not shown).

To determine the phosphoamino acid content of activin type I receptors, COS cells were cotransfected with ActRIIB and ActRI, or ActRIIB and ActRIB, metabolically labeled with [^{32}P]orthophosphate, and the activin type I receptor immunoprecipitates were subjected to two-dimensional phosphoamino acid analysis. As shown in Fig. 3C, both activin type I receptors contain predominantly phosphoserine, with a trace amount of phosphothreonine; phosphotyrosine was not detectable. This result is consistent with the predicted specificity of the activin receptors.

Binding properties of type II activin receptors lacking the kinase domain

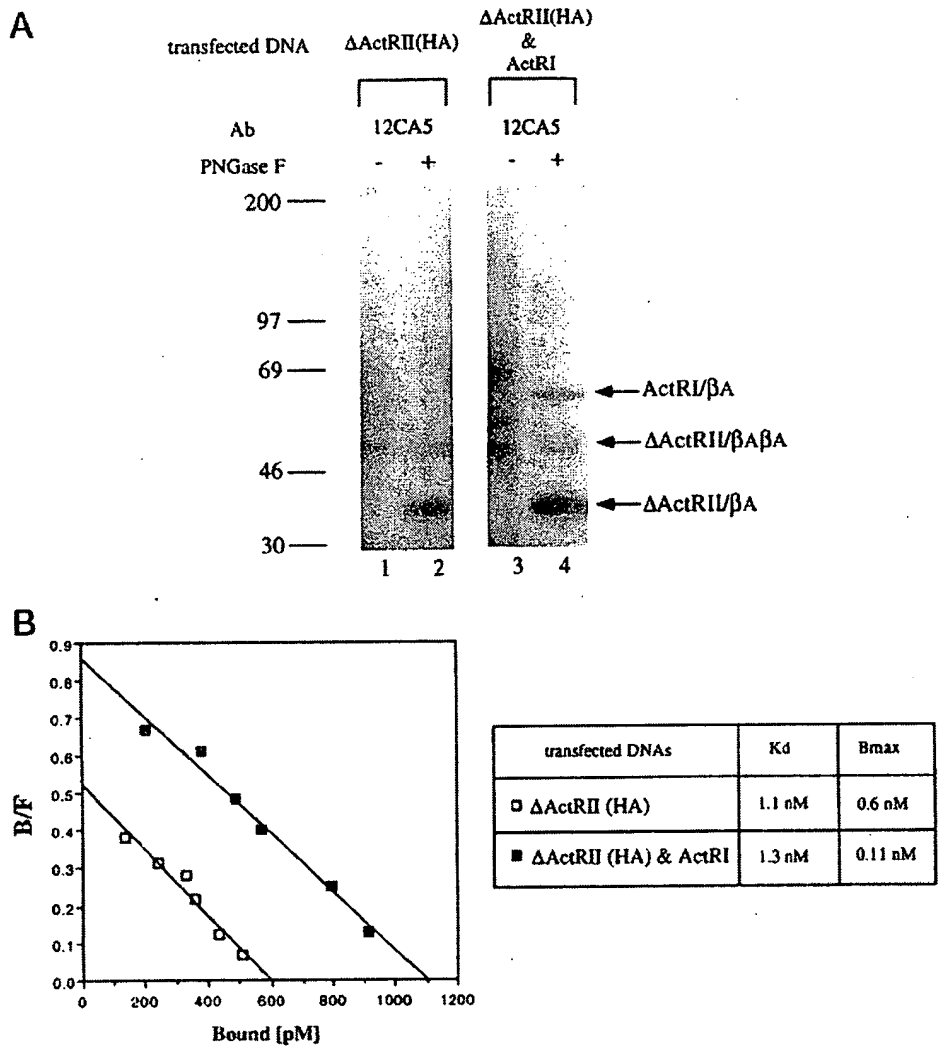
Truncated and kinase-inactive ActRIIB and TGFβRII are reported to act as dominant negative mutants, suggesting that complex formation is a prerequisite for signaling (30–32). To determine whether a truncated form of ActRII lacking the serine/threonine kinase domain and tagged with the HA epitope [ΔActRII(HA)] forms a complex with intact activin type I receptors, affinity labeling and immunoprecipitation studies were conducted. COS cells were either transfected with ΔActRII(HA) alone or cotransfected with ActRI, affinity labeled with [^{125}I]activin A, and immunoprecipitated with the 12CA5 monoclonal antibody directed against the HA epitope tag. Cells transfected with ΔActRII(HA) gave a diffuse affinity-labeled complex (Fig. 4A, lane 1), which was reduced to two distinct bands of approximately 38 and 52 kDa when immunoprecipitates were treated with peptide *N*-glycosidase F, which removes all *N*-linked carbohydrates (Fig. 4A, lane 2). The 38- and 52-kDa bands are most likely ΔActRII(HA) bound with either one monomeric subunit of activin or two monomeric subunits (or dimer) of activin, respectively. The two forms, presumably containing either one or two activin monomer subunits, were seen when wild-type ActRII was overexpressed in COS cells (19). In cells cotransfected with ΔActRII(HA) and ActRI, an additional prominent ~68-kDa band was coprecipitated with the ΔActRII(HA) affinity-labeled complex by HA antibody (Fig. 4A, lane 3). This band was reduced to ~63 kDa when immunoprecipitates were deglycosylated with peptide *N*-glycosidase F (Fig. 4A, lanes 4). The identity of this complex as ActRI was confirmed using the ActRI antiserum ARI-5536 for immunoprecipitation. The same association was seen when ΔActRII(HA) was cotransfected with ActRIB (not shown). In cotransfected cells, minor ~70- and ~95-kDa bands could also be detected, but their identities remain to be determined.

Competition experiments showed that the K_d for [^{125}I]activin A binding to ΔActRII(HA)-transfected COS cells is 1.1 nM (Fig. 4B), indicating that the binding affinity of ΔActRII(HA) is only slightly lower than that of cloned full-length type II activin receptors (200–800 pM) (7, 8, 11, 12). Thus, the kinase domain of ActRII is dispensable for high affinity binding to activin. When ActRI is cotransfected with ΔActRII(HA), the K_d of the binding of [^{125}I]activin A is 1.3 nM (Fig. 4B). This indicates that, as was observed for wild-type ActRII, the presence of ActRI did not markedly change the binding affinity of ΔActRII(HA) for activin, supporting the idea that type II receptor is the major determinant of binding affinity to the ligand (8, 9). It was previously reported that ActRI decreased the binding of ActRII to activin (8, 10), although the significance of this inhibition is not known. In contrast, ActRI did not decrease activin binding to ΔActRII(HA) and, indeed, increased the total binding approximately 2-fold (Fig. 4B). Because the intensity of the ΔActRII(HA) cross-linked complex was not affected by the presence of ActRI, the increased binding is probably due to activin binding to ActRI.

Dominant negative effects of kinase-deficient activin receptors

To investigate whether ΔActRII(HA) could actually act as a dominant negative mutant to block the functional responses of activin, we used the 3TP-Lux reporter construct that is induced by activin (10, 15). In CHO cells, 3TP-Lux transcriptional activation was observed by activin treatment, as described previously (10) (Fig. 5A). Transient expression of ΔActRII(HA) did not affect the basal level of luciferase activity, but significantly suppressed activin-induced 3TP-Lux transcriptional activation in a dose-dependent manner (Fig. 5A). These results show that ΔActRII(HA) can actually act as a dominant negative mutant by forming a nonfunctional complex with endogenous activin receptors, most likely ActRIB in CHO cells, because CHO cells use ActRIB as an activin type I receptor (Fig. 1A). Similar dominant negative effects of ΔActRII(HA) were observed in K562 cells (Fig. 5B). To test the specificity of this dominant negative effect, we attempted to rescue the mutant phenotype by exogenous wild-type ActRII. When wild-type ActRII cDNA was cotransfected with ΔActRII(HA) cDNA in K562 cells, basal luciferase activity was slightly elevated (Fig. 5B). Activin treatment of the cells cotransfected with ΔActRII(HA) and wild-type ActRII restored the transcriptional responsiveness that was suppressed by ΔActRII(HA) (Fig. 5B), indicating that the effect of ΔActRII(HA) is dominant negative.

We next compared the inhibitory effects of kinase-deficient truncated activin type I receptors (ActRI and ActRIB). Previous reports indicated that ActRIB significantly induced 3TP-Lux transcriptional activation, whereas the effect of ActRI on this transcriptional response was limited (10, 33). Like ΔActRII(HA), ΔActRIB(*myc*) and ΔActRI(*myc*) contain the extracellular domains and transmembrane domains, but lack the entire kinase domains. ΔActRIB(*myc*), similar to ΔActRII(HA), suppressed activin-induced 3TP-Lux transcriptional activation in a dose-dependent manner in both CHO and K562 cells (Fig. 6, A and B). Wild-type ActRIB rescued the effects of ΔActRIB(*myc*), indicating that ΔActRIB(*myc*) acts as a dominant negative mutant. Dominant negative effects similar to those of the ΔActRIB(*myc*), which contains five *myc* epitopes, were observed using a non*myc*-tagged ΔActRIB as well as ΔActRIB constructs containing between one and four *myc* epitope tags (not shown). In contrast, ΔActRI(*myc*) did not show any dominant negative activity on activin-induced 3TP-Lux transcriptional activation in either CHO or K562 cells (Fig. 6, *A* and *B*). Similar results were obtained using other ΔActRI constructs that were either non-*myc* tagged or contained between one and four *myc* epitopes attached to the C-terminus of the receptor (not shown).



transfected DNAs	Kd	Bmax
□ ΔActRII (HA)	1.1 nM	0.6 nM
■ ΔActRII (HA) & ActRI	1.3 nM	0.11 nM

FIG. 4. Complex formation of truncated ActRII with ActRI. A, COS cells transfected with either ΔActRII(HA) alone or together with ΔActRII(HA) were bound with 2 nM [^{125}I]activin A and cross-linked with DSS. The detergent-solubilized fractions were immunoprecipitated with the monoclonal antibody 12CA5 directed against the HA epitope and resolved by SDS-PAGE under reducing conditions. In lanes 2 and 4, immunoprecipitates were deglycosylated by treatment with peptide *N*-glycosidase F (PNGase F). M_r markers, in kilodaltons, are indicated on the *left*; the positions of ΔActRII bound with either one or two activin βA-subunits (ΔActRII/βA or ΔActRII/βAβA, respectively) and ActRI bound with one activin βA-subunit (ActRI/βA) are indicated on the *right*. B, Binding of [^{125}I]activin A to transfected COS cells. COS cells transfected either with ΔActRII(HA) (□) alone or together with ActRI (■) were bound with various concentrations of [^{125}I]activin A in the absence or presence of 40 nM unlabeled activin A. Cells were washed and solubilized in 0.5 N NaOH, and cell-associated radioactivity was quantitated. The values are the means of triplicate determinations. Scatchard plot analysis is shown. The binding capacity (Bmax) and K_d values are shown in the table. B/F, Bound/free ratio.

To confirm that ΔActRI(*myc*) is expressed at a level comparable to ΔActRIB(*myc*) in transfected cells, cell surface proteins of K562 cells transfected with ΔActRIB(*myc*) and ΔActRI(*myc*) were labeled with biotin, and the detergent-soluble cell lysate was precipitated using *myc* monoclonal antiserum 9E10. Comparable levels of expression of 31- to 35-kDa protein were detected in cells transfected with either ΔActRI(*myc*) or ΔActRIB(*myc*) (Fig. 6C). The protein size is consistent with the expected mol wt of glycosylated ΔActRIB(*myc*) or ΔActRI(*myc*). The 55-kDa protein that was detected in mock as well as receptor-transfected cells is a nonspecifically biotinylated protein (Fig. 6C). Although we cannot confirm that the non-*myc*-tagged ΔActRI was expressed, the non-*myc*-tagged ActRIB was expressed and functioned as a dominant negative receptor. Furthermore, receptor expression did not appear to be related to the number of *myc* epitopes attached to the C-terminus of either ΔActRI or ΔActRIB, as other *myc*-tagged constructs containing between one and five *myc* epitopes were also detected (not shown). These results indicate that ΔActRI(*myc*), even when expressed at a level comparable to that of ΔActRIB(*myc*), could not block the activin-dependent 3TP-Lux transcriptional response.

Discussion

Activin, similar to TGFβ, signals through heteromeric receptor complexes composed of type I and type II receptors

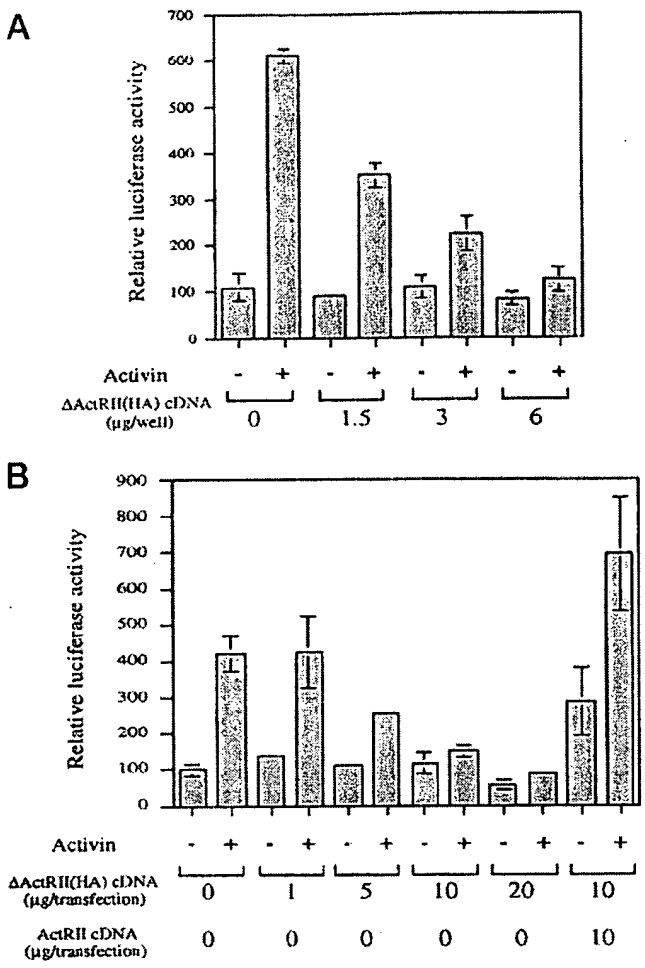


FIG. 5. Dominant negative effects of ΔActRII(HA) in CHO and K562 cells. A, ΔActRII(HA) suppressed 3TP-Lux transcriptional activation by activin in CHO cells. CHO cells in six-well dishes were cotransfected with p3TP-Lux, RSV-βgal, and various amounts of ΔActRII(HA) cDNA, and treated with or without 2 nM activin A for 24 h. The luciferase activity of each cell lysate was measured and normalized to the β-galactosidase activity. The values represent the SEM of triplicate determinations. B, ΔActRII(HA) suppressed 3TP-Lux transcriptional activation by activin in K562 cells. K562 cells were cotransfected with p3TP-Lux, CMV-βgal, and various amounts of ΔActRII(HA) cDNA. For rescuing the suppression by ΔActRII(HA), the same amounts of ΔActRII(HA) cDNA and wild-type ActRII cDNA were cotransfected. After 24 h of transfection, cells were divided and treated without or with 800 pM activin A for 12 h. The luciferase activity of each cell lysate was measured and normalized to the β-galactosidase activity. The values represent the SEM of triplicate determinations.

(8, 10, 15, 34). Two activin type II receptors (ActRII and ActRIIB) and two activin type I receptors (ActRI and ActRIB) are known (8–10). Association of type I and type II receptors is required to mediate the biological responses of activin (15). Other type I receptors (ALK-1, -5, and -6) also form complexes with ActRII, but have not been shown to elicit cellular responses (10, 35). Antisera raised against ActRI, ActRIB, or ActRII can immunoprecipitate both type I and type II affinity-labeled complexes from the activin-responsive cell line K562 in which activin induces hemoglobin synthesis, demonstrating a tight association of type I and type II receptors in this cell line. In other activin-responsive cell lines such as CHO, in which activin inhibits cell growth and regulates steroidogenesis (36), ActRI antiserum immunoprecipitates the activin affinity-labeled complex very weakly, whereas ActRII and ActRIB antisera can immunoprecipitate both type I and type II complexes. Thus, cell type-specific expression of type I receptors and their association with ligand-binding type II receptors could be responsible for the diverse cell-specific effects of activin. ActRIB appears to be the authentic activin type I receptor, because it is expressed in all activin-responsive cells studied (not shown) and mediates the signaling of growth inhibition and 3TP-Lux induction (33). It must be determined whether ActRI has its own signaling capability different from that of ActRIB. It is worth while to note that BMP-2 and BMP-7 can induce a transcriptional response through BMPRII and ActRI complexes, whereas the 3TP-Lux transcriptional effects of activin through ActRI are very weak even in the presence of ActRII (10, 37).

The stoichiometry of activin receptor complexes and the receptor interactions with activin are not delineated. One report suggests that TGFβ induces the formation of a heterotetramer containing two molecules each of TGFβRI and TGFβRII (38). Formation of a functional activin receptor complex also appears to occur through binding of activin to type II and type I receptors. This may suggest a more general ligand receptor stoichiometry for activin and other members of the TGFβ superfamily, which differs from the stoichiometry of growth factor-RTK complexes. In addition, the ligand requirements for the RSK interaction must be defined, such as determining whether the binding sites of activin to type I and type II receptors are similar or different.

Activin type I receptors were found to be phosphoproteins *in situ* and underwent ActRIIB kinase-dependent phosphorylation in transfected COS cells. Thus, it is possible that activin type I receptors act as substrates for type II receptors. Phosphoamino acid analysis showed that activin type I receptors, immunoprecipitated from metabolically labeled cells, contained predominantly P-Ser, with a small amount of P-Thr, but no detectable P-Tyr. Similar results were found for the autophosphorylation of ActRII (19) and are consistent with the predicted specificity of activin receptors. One report, however, showed that purified ActRIIB from P19 cells could both autophosphorylate and phosphorylate exogenous substrates on tyrosine residues as well as serine/threonine residues (39).

Type II kinase-dependent type I phosphorylation was found to be independent of added activin in this study. This may result from the high expression levels of receptor and/or receptor aggregation in transfected COS cells. COS cells cotransfected with various combinations of type IIB and type I receptors showed approximately 150,000 high affinity binding sites/cell. This is 30–100 times more activin-binding sites than observed in P19 or other activin-responsive cells (40, 41). Similarly, ligand-independent TGFβRI phosphorylation by TGFβRII was recently reported using the COS cell system or a baculovirus expression system in which receptors were overexpressed at high levels (42, 43). In activin-responsive cells such as P19 or CHO cells, we are as yet unable to detect ligand-dependent type I receptor phosphor-

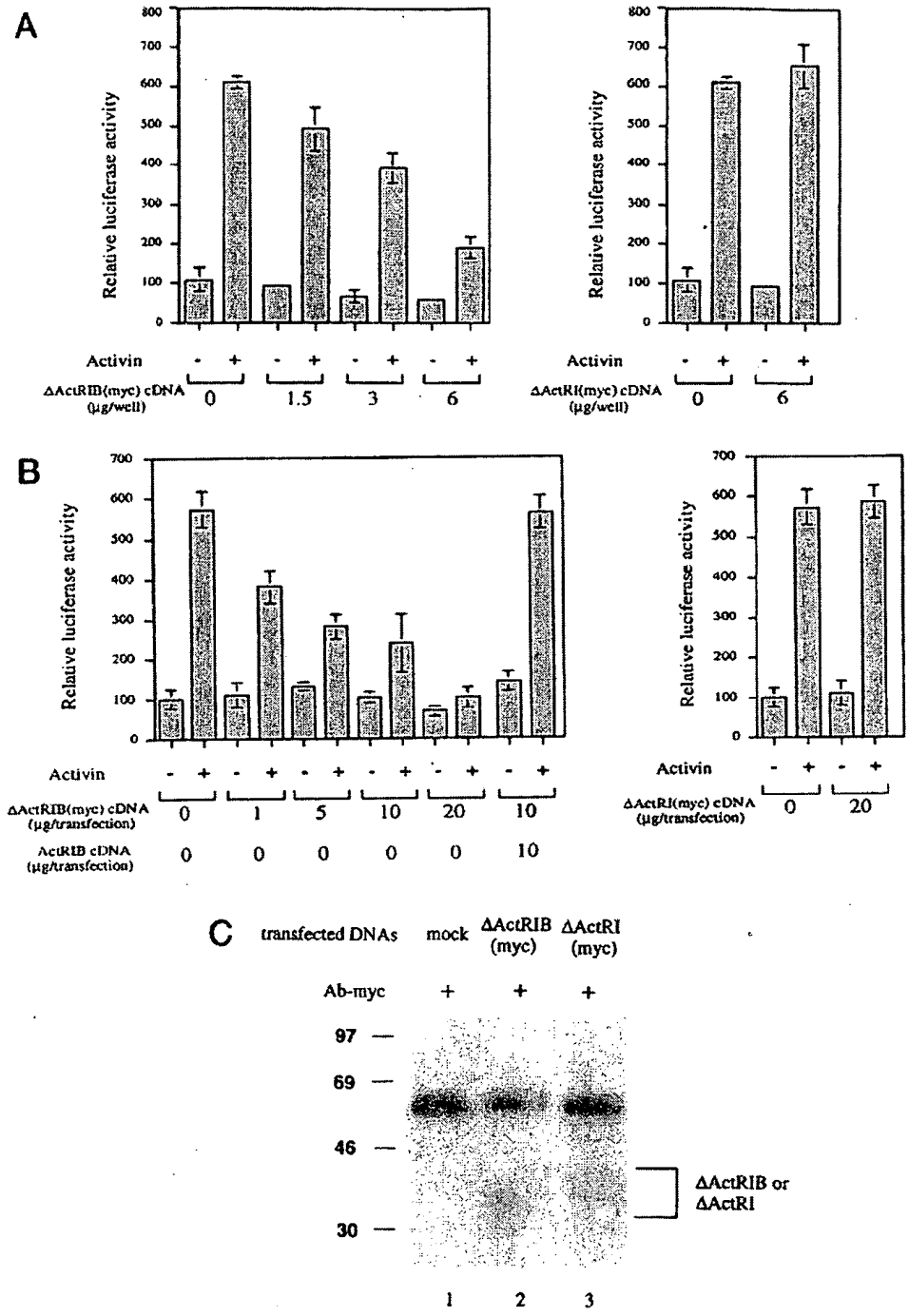


FIG. 6. Dominant negative effects of ΔActRIB(*myc*) and ΔActRI(*myc*) in CHO and K562 cells. A, ΔActRIB(*myc*), but not ΔActRI(*myc*), suppressed 3TP-Lux transcriptional activation by activin in CHO cells. CHO cells in six-well dishes were cotransfected with p3TP-Lux, RSV-βgal, and various amounts of either ΔActRIB(*myc*) or ΔActRI(*myc*) cDNA, and treated with or without 2 nM activin A for 24 h. The luciferase activity of each cell lysate was measured and normalized to the β-galactosidase activity. The values represent the SEM of triplicate determinations. B, ΔActRIB(*myc*), but not ΔActRI(*myc*), suppressed 3TP-Lux transcriptional activation by activin in K562 cells. K562 cells were cotransfected with p3TP-Lux, CMV-βgal, and various amounts of either ΔActRIB(*myc*) or ΔActRI(*myc*) cDNA. For rescuing the suppression by ΔActRIB(*myc*), the same amount of ΔActRIB cDNA and wild-type ActRIB cDNA was cotransfected. After 24 h of transfection, cells were divided and treated without or with 800 pM activin A for 12 h. The luciferase activity of each cell lysate was measured and normalized to the β-galactosidase activity. The values represent the SEM of triplicate determinations. C, Cell surface expression of ΔActRIB(*myc*) and ΔActRI(*myc*) in transfected K562 cells. K562 cells were mock transfected or transfected with either ΔActRIB(*myc*) or ΔActRI(*myc*) cDNA. Cell surface proteins were labeled by biotin as described in *Materials and Methods*. Biotin-labeled ΔActRIB(*myc*) or ΔActRI(*myc*) was immunoprecipitated with *myc* monoclonal antibody 9E10, resolved by 12% acrylamide SDS-PAGE, transferred to nitrocellulose, and visualized using the ECL system (Amersham). M_r markers, in kilodaltons, are indicated on the *left*; the positions of ΔActRIB(*myc*) and ΔActRI(*myc*) are indicated on the *right*.

ylation reliably due to the low number of endogenous receptors (not shown).

The kinase domains and kinase activities of both ActRI and ActRII are dispensable for complex formation (7). Truncated ActRII forms a complex with type I receptors (this study), and truncated type I receptors form a complex with ActRII (9). This observation suggests a mechanism by which truncated ActRII can act as a dominant negative mutant to block the effects of activin on *Xenopus* embryonic mesoderm formation (30, 31) as well as to block 3TP-Lux induction in CHO and K562 cells (this study). Because ActRII kinase activity is required for type I phosphorylation, the truncated form of ActRII probably forms a nonfunctional complex with type I receptors and blocks activin action. Thus, it is likely that phosphorylation of type I receptors in a type II kinase-dependent manner is required for activation and signal transduction. As predicted by this model, ActRIIB(K217R) also exerts dominant negative effects to block activin-induced 3TP-Lux transcriptional activity in CHO and K562 cells (not shown). Through this mechanism, dominant negative activin receptors have been shown to be useful tools to study the physiological roles of activin *in situ*, such as the induction of mesoderm formation in early development (23, 24) and the regulation of gene transcription (this study).

Similar to truncated ActRII, truncated ActRIB(ΔActRIB) also acts as a dominant negative mutant to block the activin-induced transcriptional response. Because type I receptors are not required for type II receptor phosphorylation, ΔActRIB presumably prevents endogenous ActRIB from phosphorylating downstream substrate(s) by combining with endogenous receptors to form a nonfunctional receptor complex. Surprisingly, ΔActRI fails to block the activin-induced 3TP-Lux transcriptional response. Because ΔActRI is expressed at a level comparable to that of ΔActRIB, the affinity of ΔActRI for the endogenous activin receptor complex may be lower than that of ΔActRIB. Alternatively, it is also possible that ΔActRI is able to block activin responses other than 3TP-Lux transcription. In accord with the hypothesis that ActRIB and ActRI have different signaling capabilities, ΔActRIB and ΔActRI block different marker genes in *Xenopus* embryos (44) (Bhushan, A., and W. W. Vale, manuscript in preparation).

A receptor activation model has been proposed for the TGFβ receptor system (45). TGFβ binds type II receptors first, then the TGFβ-type II complexes associate with and phosphorylate type I receptors to trigger downstream signaling. Type II receptors exhibit a basal level of autophosphorylation that is independent of type I receptors. Type I receptors, when they form a complex with type II receptors, act as substrates for constitutively active type II receptors. Like the TGFβ receptor system, it is likely that the activin type I receptors could act as substrates for activin type II receptors, and type I receptors could phosphorylate downstream targets. Neither the intracellular substrates for activin receptors nor the downstream signaling mechanisms are well characterized. Recently, the specific interaction between type I receptors and the immunophilin FKBP-12, which is a binding protein for FK506, has been reported (46). FKBP-12 could be either the substrate for activin receptors or an adaptor protein that combines the receptors with the next substrate; however, a functional role for FKBP12 in type I receptor-mediated RSK signaling has yet to be reported. In any case, activin signal transduction through two distantly related transmembrane serine kinases is likely to be fundamentally different from the signaling mechanism of receptor tyrosine kinases (16–18).

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